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Chlorophyll *b* reduction during senescence of barley seedlings

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Abstract. During senescence of flowering plants, only breakdown products derived from chlorophyll *a* were detected although *b* disappears, too (Matile et al., 1996, Plant Physiol 112: 1403–1409). We investigated the possibility of chlorophyll *b* reduction during dark-induced senescence of barley (*Hordeum vulgare* L.) leaves. Plastids isolated from senescing leaves were lysed and incubated with NADPH. We found 7^1-hydroxy-chlorophyll *a*, 7^1-hydroxy-chlorophyllide *a*, and, after incubation with Zn-pheophorbide *b*, also Zn-7^1-hydroxy-pheophorbide *a*, indicating activity of chlorophyll(ide) *b* reductase. The highest activity was found at day 2 of senescence when chlorophyll breakdown reached its highest rate. Chlorophyllase reached its highest activity under the same conditions only at days 4–6 of senescence. Based on the chlorophyll *b* reductase activity of plastids at day 2.5 of senescence (=100%), the bulk of activity (83%) was found in the thylakoids and only traces (5%) in the envelope fraction. Chlorophyll *b* reduction is considered to be an early and obligatory step of chlorophyll *b* breakdown.

Key words: Chlorophyllase – Chlorophyll *b* reductase – *Hordeum* – 7^1-*Hydroxy-chlorophyll a* – Leaf senescence

Introduction

The light-harvesting complexes (LHCs) of higher plants and green algae contain chlorophyll (Chl) *b* besides Chl *a*. The pathway of biosynthesis of Chl *b* from Chl *a* or a Chl *a* precursor is not yet known in detail (Porra 1997; Rüdiger 1997); it implies the incorporation of dioxygen into the methyl group at C-7 of the macrocycle (Schneegurt and Beale 1992; Porra et al. 1994). The gene encoding an oxygenase responsible for Chl *b* biosynthesis in *Chlamydomonas rheinhardtii* was recently identified (Tanaka et al. 1998). The 7^1-OH-Chl *a* was proposed as a possible intermediate in Chl *b* formation from Chl *a* (Porra et al. 1994; for the structures see Fig. 1). The reverse reaction, reduction of the formyl group at C-7 of Chl *b* to the methyl group of Chl *a*, can be achieved as a chemical reaction (Scheumann et al. 1996a) or as an enzymatic reaction (Ito et al. 1993, 1994, 1996). Both the chemical and the enzymatic reaction proceed via the 7^1-OH intermediate; the first step of the enzymatic reaction requires NADPH and the second step reduced ferredoxin (Scheumann et al. 1996b, 1998). Chlorophyll *a* oxygenation and Chl *b* reduction form the "chlorophyll cycle" (Ito et al. 1996).

The physiological significance of the conversion of Chl *b* to Chl *a* is not yet known. On the one hand, Ito et al. (1993) proposed a role in acclimation of plants from low-light to high-light conditions, when fewer LHC II complexes and hence less Chl *b* than Chl *a* is needed, and during the early stages of greening. On the other hand, only degradation products of Chl *a* were detected in senescing barley and rape leaves while Chl *b* was degraded together with Chl *a* (Kräutler et al. 1991; Mühlecker et al. 1993). In contrast, pigment catabolism in the green alga *Chlorella protothecoides* yields degradation products from both Chl *a* and Chl *b* (Engel et al. 1991; Iturraspe et al. 1994). Gossauer and Engel (1996) proposed that the conversion of Chl *b* to Chl *a* should precede Chl degradaton in higher plants but not in algae. We demonstrate here a temporary increase in the activity of Chl *b* reductase in senescing barley leaves, indicating a physiological role for this enzyme in Chl degradation.

Abbreviations: Chl = chlorophyll; Chlide = chlorophyllide; Pheide = pheophorbide

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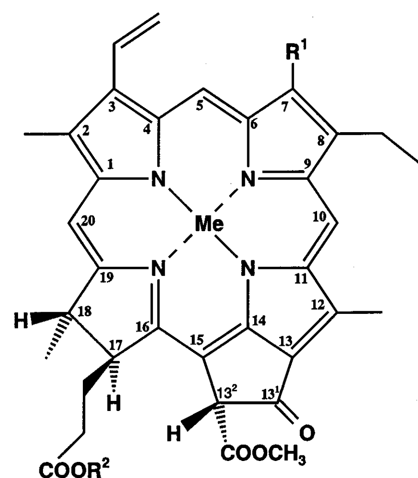
Materials and methods

Pigment preparation. Zn-Pheophorbide (Pheide) *b* was prepared as described previously (Helfrich et al. 1994) and Zn-7^1-OH-Pheide *a*,



	Me	R^1
Zn-pheophorbide *a*	Zn	CH_3
Zn-pheophorbide *b*	Zn	CHO
Zn-7^1-OH-pheophorbide *a*	Zn	CH_2OH
chlorophyllide *a*	Mg	CH_3
chlorophyllide *b*	Mg	CHO
7^1-OH-chlorophyllide *a*	Mg	CH_2OH
pheophorbide *a*	2H	CH_3
pheophorbide *b*	2H	CHO
7^1-OH-pheophorbide *a*	2H	CH_2OH

Fig. 1. Chemical structures of educts and products of Chl *b* reductase. The central metal (*Me*) can be zinc or magnesium in the enzyme reaction; in some experiments, magnesium was removed for product analysis. $\mathbf{R}^2$, H for the named compounds and phytol for the corresponding Chls and pheophytins

which was used as a standard substance, was synthesized according to Scheumann et al. (1996a).

Plant material. Caryopses of barley (*Hordeum vulgare* L. cv. Steffi; Dr. Ackermann, Irlbach, Germany) were germinated on moist vermiculite. For isolation of chloroplasts, the seedlings were grown in cycles of 12 h darkness/12 h white light (33 μmol photons m^{-2} s^{-1}) for 7 d. For isolation of gerontoplasts, the leaves of 7-d-old green seedlings were cut and incubated in beakers with water in darkness for 1–8 d at 25 °C.

Plastid isolation and enzyme reaction. Plastids were isolated as described for etioplasts by Eichacker et al. (1996), and the reaction was carried out according to Scheumann et al. (1998). All steps were carried out at 4 °C under dim-green light (< 10 nmol photons m^{-2} s^{-1}). In brief, the primary leaves were chopped with an Ultraturrax in isolation buffer (50 mM Hepes-KOH, pH 8.0, containing 0.4 M sorbitol and 2 mM EDTA). Large cell debris was removed by filtration through gauze, and the crude plastids were collected by centrifugation (3800 *g*, 1.5 min). Plastid purification was then achieved by centrifugation (4100 *g*, 7 min) with a step gradient of Percoll (40%/80% for chloroplasts and gerontoplasts) in the same buffer. Intact plastids, which were found in the boundary of the gradient (40% to 80%) were collected, suspended in the same buffer without EDTA, and counted in a hematocytometer after appropriate dilution (usually 1:200).

Samples of 8.5×10^7 plastids were lysed in 100 μl of 50 mM Hepes-KOH (pH 8.0), containing 10 mM $MgCl_2$ (reaction buffer) and incubated with 2 mM NADPH at 28 °C for 90 min in darkness. In some experiments, Zn-Pheide *b* was added at the beginning of the incubation. The reaction was stopped with acetone (final volume 1 ml containing 80% acetone), and the esterified pigments were extracted into *n*-hexane (2 × 500 μl). For extraction of the non-esterified pigments, the lower phase of the hexane extraction was mixed with 500 μl ethyl acetate and 500 μl of 20% aqueous NaCl. The extraction with 500 μl ethyl acetate was repeated. Absorption spectra of the esterified and non-esterified pigments were recorded. The solvents were then evaporated and the residue was dissolved in 30 μl acetone. Aliquots of 10 μl were used for HPLC analysis.

Analysis by HPLC. The esterified pigments were analyzed by HPLC with a reverse phase column (250 mm long, 4 mm i.d.; C-18 Hypersil ODS 5 μm; Shandon) at a flow rate of 1.3 ml min^{-1}. The separation started with 70% acetone/30% H_2O for 2 min, followed by linear gradients to 82% acetone within 2 min, to 88% acetone within 11 min, and to 100% acetone within 4 min. The non-esterified pigments were analyzed using a column (250 mm long, 4.6 mm i.d.) filled with Rosil C-18 (Grom Analytik, Herrenberg, Germany), 5 μm, and a flow rate of 1.5 ml min^{-1}. The pigments were eluted with gradients from 50% acetone/50% H_2O, adjusted to pH 3.5 with acetic acid, to 63% acetone within 23 min, and to 100% acetone within 6 min.

The columns were connected to a diode-array spectrophotometer (Tidas, J&M, Aalen, Germany) and a spectrofluorimetric detector (RF-551; Shimadzu). An absorption spectrum from 350 nm to 750 nm was recorded every second.

Quantification of the pigments. Zn-7^1-Hydroxy-pheophorbide *a*, 7^1-OH-Pheide *a* and 7^1-OH-Chl *a* were quantified by integrating the corresponding peaks of the HPLC analysis after calibration with a known amount of a standard substance. The amounts of Chl *a* and Chl *b* were determined from their absorption spectrum in *n*-hexane. The molar extinction coefficients (E) were calculated from the extinction coefficients given by French (1960) for Chl *a* and Chl *b* in diethylether and the following formula derived:

$$\mathrm{Chl}\,a = 11.26 \times E_{662} - 1.10 \times E_{644} - 0.085 \times E_{624}\ (\mathrm{nmol\ ml^{-1}})$$

$$\mathrm{Chl}\,b = 18.20 \times E_{644} - 2.96 \times E_{662} - 0.45 \times E_{624}\ (\mathrm{nmol\ ml^{-1}})$$

Chlorophyllide (Chlide) *a* and Chlide *b* were calculated from their absorption spectrum in ethyl acetate. Using the molar extinction coefficients for Chlide *a* and *b* in 80% acetone given by Scheer (1988) for the red maxima, the extinction coefficient at the maximum wavelength of the other pigment was determined in ethyl acetate and the following formula derived:

$$\mathrm{Chlide}\,a = E_{663} \times 12.8 - E_{646} \times 2.3\ (\mathrm{nmol\ ml^{-1}})$$

$$\mathrm{Chlide}\,b = E_{646} \times 21.8 - E_{663} \times 4.5\ (\mathrm{nmol\ ml^{-1}})$$

Preparation of thylakoid and envelope membranes. For preparation of thylakoid and envelope membranes from gerontoplasts according to Matile et al. (1992) and Matile and Schellenberg (1996), barley seedlings (*Hordeum vulgare* L., cv. Wintergerste "Baraka") were grown under a 12 h/12 h photoperiod for 10 d, and leaves (about 20 g FW) were subsequently placed in darkness for 1.5–2.5 d. The preparation of intact plastids by lysis of protoplasts was achieved as described by Matile et al. (1992), but using Tris-HCl instead of Tricine-KOH and a step gradient of 20%/80% Percoll instead of pelleting the plastids from 20% Percoll. The plastids were lysed and the membranes fractionated according to Matile and Schellenberg (1996) with the following modifications: the lysis buffer contained 5 mM Mops-Tris (pH 7.6), 2 mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol, and the step gradient was composed of the same buffer with 1.2 M, 0.96 M and 0.45 M sucrose. After centrifugation (90 000 *g*, 30 min), the thylakoids were on top of the 1.2 M sucrose layer and the envelopes in the boundary 0.96/0.45 M sucrose. Both membrane fractions were quantitatively collected, washed with the lysis buffer and resuspended in the same buffer. An aliquot corresponding to 1×10^8 plastids per sample was used for the enzyme reaction. As markers, Chl was used for thylakoid and Pheide *a* oxygenase for envelope membranes.

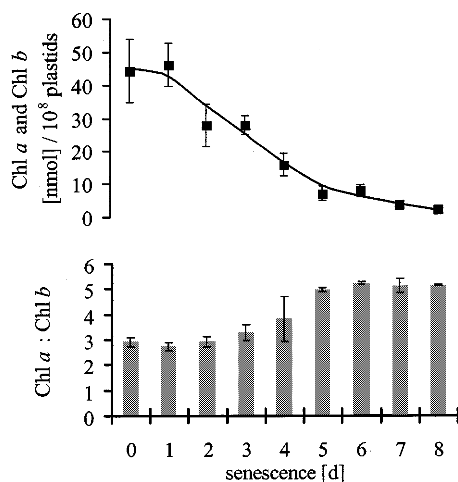


Fig. 2. Decrease of total Chl (*upper panel*) and change of the ratio Chl *a*:Chl *b* (*lower panel*) during dark-induced senescence of barley leaves. Plastids were isolated from the leaves at the indicated time points and the pigments were extracted with acetone. The amounts of Chl *a* and Chl *b* were determined by spectrophotometry. The values are means of three independent experiments ±SD

Results

For investigation of the suspected reduction of Chl *b* during senescence, we kept barley seedlings under light-dark cycles for one week, cut the shoots and placed them in darkness according to Matile et al. (1987). Samples were harvested every day, and plastids were isolated, lysed by freezing and thawing, incubated for 90 min in vitro and then used for analysis. This procedure allowed us to determine pigment content and enzyme activities under precisely identical conditions.

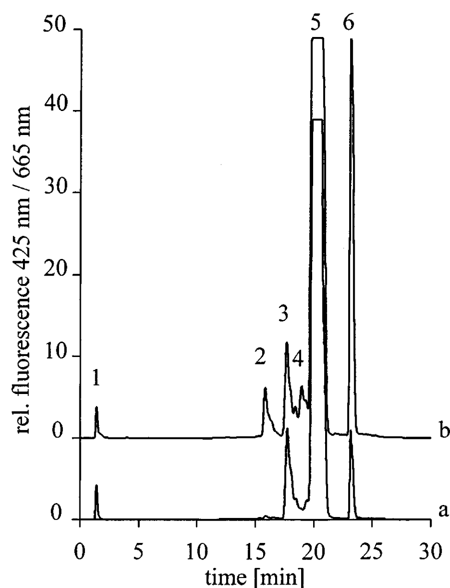


Fig. 3. Analysis by HPLC of the esterified pigments of chloroplasts (*curve a*) and gerontoplasts isolated from barley leaves which had been kept in darkness for 2 d (*curve b*). The lysed organelles were incubated in darkness for 90 min with NADPH. The pigments were quantitatively transferred into *n*-hexane and analyzed by HPLC. The excitation was set at 425 nm, and the fluorescence emission was detected at 665 nm. The pigments were identified by their retention time (comparison with authentic compounds) and absorption spectrum (see Fig. 4) as follows: *1*, Chlide *a*; *2*, 7^1-OH-Chl *a*; *3*, Chl *b*; *4*, 13^2-OH-Chl *a*; *5*, Chl *a*; *6*, pheophytin *a*

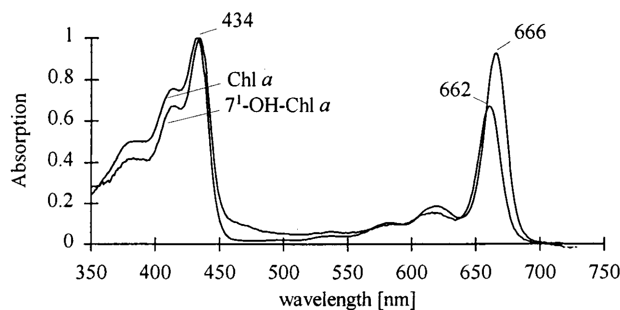


Fig. 4. Absorption spectra of the pigments in the peaks 2 and 5 of Fig. 3. The spectra were normalized to the Soret band at 434 nm. The pigment in peak 2 with λ_{max} = 662 nm is 7^1-OH-Chl *a*; the pigment in peak 5 with λ_{max} = 666 nm is Chl *a*

Analysis of esterified pigments. One of the criteria for senescence is the degradation of Chl. Under our experimental conditions, the total Chl content remained constant for 1 d in the dark and declined rapidly thereafter (Fig. 2). The Chl *a*:Chl *b* ratio increased during senescence from 2.9 at day 0 to 5.1 at day 8. This means that either degradation of Chl *b* was faster than that of Chl *a* or Chl *b* was transformed into Chl *a* (or a pigment with the spectral properties of Chl *a*). Incubation of the lysed plastids with NADPH for 90 min did not significantly change the decay kinetics or the Chl *a*:Chl *b* ratio (data not shown).

An intermediate of the reduction of Chl *b* to Chl *a* is 7^1-OH-Chl *a* (Ito et al. 1996; Scheumann et al. 1996b; Ohtsuka et al. 1997). The HPLC analysis of the esterified pigments revealed a new peak (peak 2, Fig. 3) in gerontoplasts, incubated with NADPH for 90 min, besides the peaks of Chl *a*, Chl *b* and pheophytin *a*. The new peak was hardly detectable in chloroplasts incubated under identical conditions (Fig. 3). The retention time (15.9 min) and the absorption spectrum with maxima at 436 and 662 nm (Fig. 4) are identical to those of 7^1-OH-Chl *a*. The absorbance ratio of Soret band to Q_y band = 1.49 determined in the HPLC solvent system, is typical of 7^1-OH-Chl *a* and is different from the ratio 1.08 for Chl *a* (Fig. 4).

The kinetics of 7^1-OH-Chl *a* formation during senescence in darkness show a pronounced peak at day 2. At day 5 and later, the same low amount as in chloroplasts was found (Fig. 5).

Analysis of non-esterified pigments. Incubation of lysed etioplasts always resulted in hydrolysis of a small amount of the Chls. Since all Chl degradation products that were isolated from senescing leaves had lost the phytol moiety of Chl (Matile et al. 1996), we also investigated the non-esterified pigments in the course of senescence. The amount of total Chlide formed during the incubation of lysed plastids can be considered as

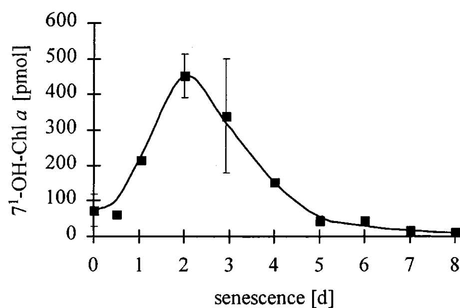


Fig. 5. Formation of 7^1-OH-Chl *a* from endogenous Chl during dark-induced senescence. Barley leaves were kept in darkness for the indicated time. The pigments of 10^8 lysed gerontoplasts were analyzed as above (see Fig. 3). Mean values ±SD

measure of the chlorophyllase activity, since the substrate is permanently present in excess. We found a small and broad maximum for shoots kept for 4–6 d in darkness (Fig. 6). No influence of NADPH was detected (data not shown).

The ratio Chlide *a*:Chlide *b* showed an increase during the first 3 d and then a decrease to the initial value until day 8 (Fig. 6). This was different from the time course of the Chl *a*:Chl *b* ratio. Furthermore, there was a marked increase in "Chlide *a*" if NADPH was present during the incubation of lysed plastids (Fig. 6). We assumed that the increase caused by NADPH indicated formation of 7^1-OH-Chlide *a* which has a similar absorption spectrum to Chlide *a* and contributes to "Chlide *a*" in the spectrophotometric determination. This assumption was then verified by HPLC (Fig. 7). The slightly acid solvent which had to be used for HPLC caused the loss of Mg from the pigments. Therefore,

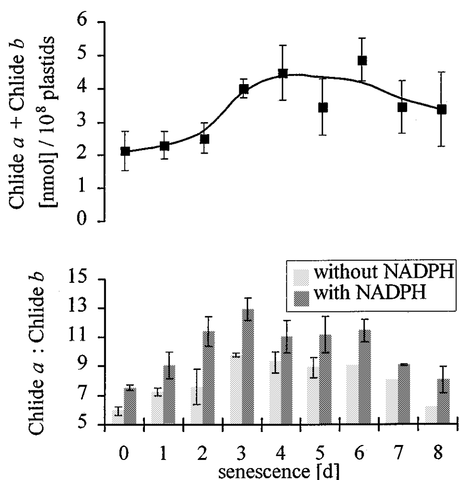


Fig. 6. Formation of Chlide *a* and Chlide *b* in gerontoplasts. Barley leaves were incubated in darkness for the indicated time. Plastids were isolated, lysed and incubated with or without NADPH for 90 min at 28 °C. The pigments from 10^8 plastids were extracted with acetone, and the esterified pigments were then transferred into ethyl acetate, and Chlide *a* and Chlide *b* were quantified by spectrophotometry. *Upper panel*: contents of total Chlide in 10^8 plastids. *Lower panel*: ratio of Chlide *a*:Chlide *b* with NADPH (*dark grey columns*) and without NADPH (*light grey columns*). The values are means of three independent experiments ±SD

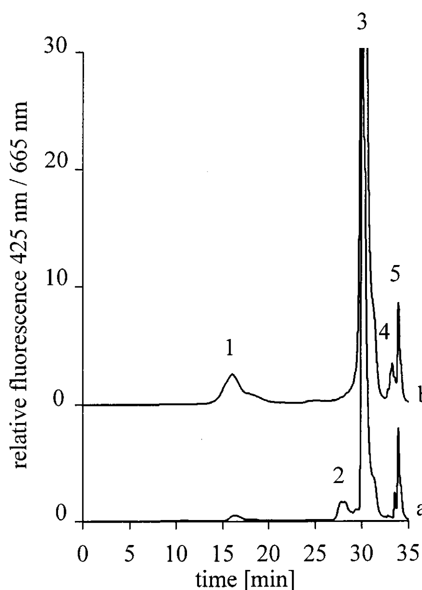


Fig. 7. Analysis by HPLC of non-esterified pigments from chloroplasts (*curve a*) and gerontoplasts (*curve b*). The treatment of barley plants, incubation of lysed plastids with NADPH, pigment extraction and transfer of the bulk of the esterified pigments were as described in Fig. 3. Subsequently, the non-esterified pigments were transferred into ethyl acetate and analyzed by HPLC. The pigments were identified by their retention time (comparison with authentic compounds) and absorption spectra as follows: *1*, 7^1-OH-Pheide *a*; *2*, Pheide *b*; *3*, Pheide *a*; *4*, pheophytin *b*; *5*, pheophytin *a*

analysis was performed with the Pheides. The pigment (peak 1 in Fig. 7) was identified by comparison of its retention time and absorption spectrum with that of authentic 7^1-OH-Pheide *a*. The time course of its formation during senescence (Fig. 8) was different from that of 7^1-OH-Chl *a*. We found a broad maximum between days 2 and 6. Since formation of 7^1-OH-Chlide *a* from Chlide *b* requires two reaction steps catalyzed by chlorophyllase and by Chl *b* reductase, the time course should be composed of the time courses of both enzyme activities. The activity maximum for Chl *b* reductase was found at days 2–3 and that of chlorophyllase at days 4–6 of senescence in darkness. The broad maximum between days 2 and 6 agrees with

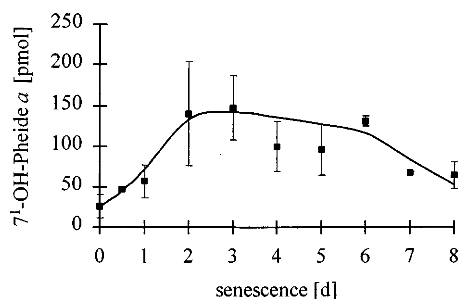


Fig. 8. Formation of 7^1-OH-Chlide *a* in gerontoplasts. For the treatment of plants and pigment extraction see Fig. 6. The peak of 7^1-OH-Pheide *a* obtained by HPLC analysis of the non-esterified pigments (see Fig. 7) was used for quantification. The values are means of three independent experiments ±SD

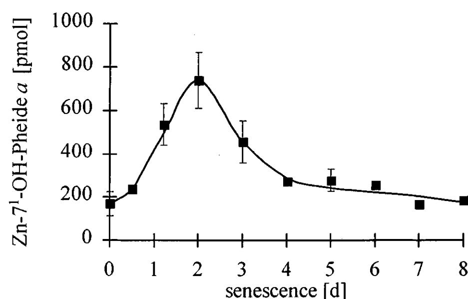


Fig. 9. Activity of Chl(ide) *b* reductase in gerontoplasts. Barley leaves were kept in darkness for 1–8 d. Plastids were isolated at the indicated time, lysed and incubated with Zn-Pheide *b* and NADPH for 90 min. The non-esterified pigments were analysed by HPLC (see Fig. 7), and the peak of Zn-7^1-OH-Pheide *a* was quantified. The values are means of two independent experiments ± SD

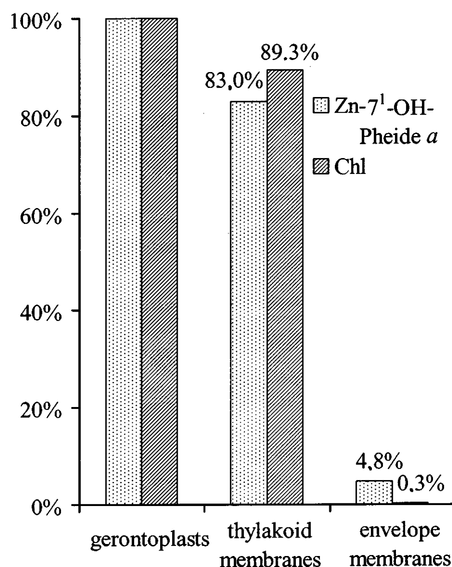


Fig. 10. Localization of Chl(ide) *b* reductase. Barley leaves were placed in darkness for 2.5 d. Plastids were isolated and membranes separated in a sucrose gradient. The activity was tested with Zn-Pheide *b* (8 nmol) and either 10^8 plastids (= gerontoplasts) or membranes prepared from 10^8 plastids (= thylakoid and envelope membranes). The amount of Chl (91 nmol) and the amount of Zn-7^1-OH-Pheide *a* (2 nmol) formed in the plastids was defined as 100%

the expected time course of a product requiring these two enzymatic steps.

Activity and localization of Chl b reductase. The preferential disappearance of Chl *b* during the appearance of 7^1-OH-Chl *a* and 7^1-OH-Chlide *a* suggests the formation of the 7^1-OH-intermediates from Chl *b*. However, formation from Chl *a* cannot completely be excluded because (i) Chl *a* also disappears at the same time and (ii) 7^1-OH-Chl(ide) *a* was also postulated as an intermediate in the biosynthesis of Chl *b* from Chl(ide) *a* (Porra et al. 1994). Therefore, we used Zn-Pheide *b* as an exogenous pigment to determine unambiguously the activity of Chl *b* reductase. We had already shown that this enzyme accepts Zn-Pheide *b* instead of Chlide *b* (Scheumann et al. 1996b) The Zn-containing reaction product can be distinguished from Mg-containing endogenous pigments by HPLC and absorption spectroscopy. As shown in Fig. 9, there was a pronounced maximum of Chl *b* reductase activity at day 2 of senescence. The kinetics coincide with the kinetics of formation of 7^1-OH-Chl *a* (Fig. 5), indicating that the latter was indeed formed from Chl *b*.

We had already shown that Chl *b* reductase is a membrane-bound enzyme (Scheumann et al. 1998). We now used the relatively high activity of the reductase in gerontoplasts to localize the enzyme. Fractionation of gerontoplasts had shown that chlorophyllase and Pheide *a* oxygenase are located in the envelope membrane (Matile and Schellenberg 1996; Matile et al. 1997). The isolated gerontoplasts were lysed and the envelope membrane separated from the thylakoid membrane by centrifugation in a sucrose gradient. Contamination of the envelope fraction with thylakoid membranes was determined by its Chl content. Both membrane fractions prepared from 10^8 plastids were incubated with Zn-Pheide *b* and NADPH under standard conditions, and the amount of Zn-7^1-OH-Pheide *a* was determined by HPLC.

Based on the activity in lysed gerontoplasts, 83% of the activity was found in the thylakoid fraction and only 4.8% in the envelope fraction (Fig. 10). This means that the bulk of Chl *b* reductase activity is in the thylakoid membrane. Since we found only 0.3% Chl in the envelope fraction, the amount of activity (4.8%) could indicate that a minor amount of the enzyme is located in the envelope membrane. However, we cannot exclude the possibility that a small part of the thylakoid membrane that had lost its Chl during senescence was partitioned into the envelope fraction and that this part of the thylakoid membrane contained the observed Chl *b* reductase activity.

Discussion

Dark-induced senescence of barley leaves has been used as an experimental system to investigate the first steps of Chl degradation (Matile et al. 1987). Later, Mühlecker et al. (1993) demonstrated that the same essential steps of Chl degradation occur in rape cotyledons during natural degreening. Thus the dark-induced senescence seems to be a good model system for senescing plants in general, at least for the question of Chl degradation. We found that the barley leaves tolerated a dark period of about 1 d without substantial loss of total Chl and that Chl degradation started thereafter in prolonged darkness. Since the major decrease was observed between days 1 and 6, the highest activity of enzymes involved in Chl degradation was expected during this period. A peak of chlorophyllase activity was indeed observed at days 4–6.

Chlorophyll *a* and Chl *b* were not degraded at the same rate. As shown by the Chl *a*:Chl *b* ratio, Chl *b* disappeared faster than Chl *a*. In contrast to natural leaf senescence which leads to a decrease in the Chl *a*:

Chl *b* ratio (Jenkins et al. 1981; Kura-Hotta et al. 1987; Hidema et al. 1992), the dark-induced senescence also results in an increase of the ratio Chl *a* to Chl *b* in *Phaseolus vulgaris* leaves (Fang et al. 1998). The authors discussed a transformation of Chl *b* to Chl *a*, but the data do not exclude different degradation pathways for Chl *a* and Chl *b*. However, our present data on the increased formation of 7^1-OH-Chl *a* point to a reduction of at least part of the total Chl *b* at the beginning of senescence. We assume that the reduction proceeds further to Chl *a* via the observed 7^1-OH-Chl *a*.

Degradation of Chl *b* via Chl *a* (or Chlide *a*) can indirectly be deduced from results with a *stay-green* mutant in which neither Chl *a* nor Chl *b* is degraded under conditions of senescence. The key enzyme of Chl degradation, Pheide *a* oxygenase, is impaired in this mutant (Vicentini et al. 1995). This enzyme accepts only Pheide *a* but not Pheide *b* (Hörtensteiner et al. 1995). These observations strongly indicate that Chl *b* degradation requires this enzyme albeit it is specific for Chl *a* derivatives. The reduction of Chl *b* must occur before the central Mg is lost because the metal-free Pheide *b* is reduced by Chl *b* reductase only to a small extent (Scheumann et al. 1996b). We concentrated our efforts here upon investigation of 7^1-OH-Chl *a* because even traces of this intermediate of the Chl *b* reduction can easily be detected in the presence of large amounts of Chls *a* and *b*. In this context, Chl *b* reductase activity signifies catalysis of the first step of Chl *b* reduction which depends on NADPH. Since the second step, reduction of 7^1-OH-Chl *a* to Chl *a*, requires reduced ferredoxin (Scheumann et al. 1998) and since there are differences in the stability of the two activities (data not shown), it is likely that there is a second enzyme (hydroxy-Chl *a* reductase) which is specific for the second reduction step.

The activity peak at day 2 of senescence indicates that Chl *b* reductase precedes chlorophyllase in the kinetics of senescence. The early peak of Chl *b* reductase activity at day 2 of senescence was confirmed with Zn-Pheide *b* as substrate. In accordance with this interpretation, formation of 7^1-OH-Chlide *a* from endogenous pigment which requires activity of both Chl *b* reductase and chlorophyllase, showed a broad maximum between days 2 and 6.

The possibility that the early peak of Chl *b* reductase in the kinetics of senescence also indicates a role for this enzyme early in the pathway of Chl degradation, is supported by its localization. We found nearly all Chl *b* reductase activity in the thylakoid membrane and only traces of activity in the envelope fraction. The thylakoid membrane is the location of Chls and hence the most probable location for beginning of their degradation. Chlorophyllase was found only in the envelope membrane and not in the thylakoid membrane (Matile et al. 1997). This means the Chls have to be transported from the thylakoid to the envelope for dephytylation. The first product of Chl *b* reduction, 7^1-OH-Chl *a*, does not bind to Chl proteins (Ohtsuka et al. 1997) and is therefore a plausible candidate for such a transport form involved in Chl *b* degradation. Furthermore, 7^1-OH-Chl *a* is accepted by chlorophyllase as a substrate (data not shown).

Our results do not exclude a role for Chl *b* reductase independent of Chl degradation. The observation that Zn-Pheide *b* and Zn-7^1-OH-Pheide *a* were reduced to Zn-Pheide *a* if infiltrated into etiolated oat leaves (Scheumann et al. 1996a) points to a high activity of Chl *b* reductase in etiolated plants. These in vivo experiments confirmed earlier in-vitro results with isolated etioplasts (Ito et al. 1993, 1994, 1996). The physiological significance of Chl *a* to Chl *b* transformation might be in this case the need for Chl *a* during the early stages of greening rather than Chl degradation. The same is probably true for experiments with intermittent light when Chl *a* is accumulated in the near absence of Chl *b* (Tzinas et al. 1987; Shimada et al. 1990). Reduction of Chl *b* to Chl *a* has yet to be shown under these conditions. The observation that etiolated bean seedlings exposed to light-dark cycles had a higher ratio of Chl *a*:Chl *b* at the end of each dark period than at the end of each light period (Akoyunoglou and Akoyunoglou 1985), indicates that the conversion of Chl *b* to Chl *a* operates each night in growing plants. In green plants, the transformation of Chl *b* to Chl *a* via 7^1-OH-Chl *a* seems to be important for acclimation from low-light to high-light conditions (Tanaka et al. 1991; Ohtsuka et al. 1997). It is not surprising, therefore, that a base level of Chl *b* reductase activity can be measured during various developmental stages. The marked increase in its activity at the beginning of dark-induced senescence shows that Chl *b* reductase must play an important role in Chl *b* breakdown.

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