

Light-independent degradation of stromal proteins in intact chloroplasts isolated from *Pisum sativum* L. leaves: requirement for divalent cations

Samuel Roulin, Urs Feller

Institute of Plant Physiology, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

Received: 3 July 1997 / Accepted: 15 October 1997

Abstract. Intact chloroplasts were isolated from mature pea (*Pisum sativum* L.) leaves in order to study the degradation of several stromal proteins in organello. Changes in the abundances of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), phosphoribulokinase (EC 2.7.1.19), glutamine synthetase (EC 6.3.1.2) and ferredoxin-dependent glutamine:α-ketoglutarate aminotransferase (glutamate synthase; EC 1.4.7.1) were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie-staining of the gels or immunoblotting using specific antibodies for the different enzymes. Degradation of several stromal proteins was strongly stimulated when intact chloroplasts were incubated in the light in the presence of dithiothreitol. Since free radicals may artificially accumulate in the chloroplast under such conditions and interfere with the stability of stromal proteins, the general relevance of these processes remains questionable. In the absence of light, proteolysis proceeded slowly in isolated chloroplasts and was not stimulated by dithiothreitol. Inhibition by ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline or excess zinc ions as well as the requirement for divalent cations suggested that a zinc-containing metalloprotease participated in this process. Furthermore, light-independent degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoribulokinase was enhanced in chloroplasts isolated from leaves in which senescence was accelerated by nitrogen starvation. Our results indicate that light-independent stromal protein degradation in intact chloroplasts may be analogous to proteolysis that occurs in intact leaves during senescence.

Key words: Chloroplast – Nitrogen remobilization – *Pisum* – Proteolysis – Senescence – Stromal enzymes

Introduction

Numerous examples of protein degradation in chloroplasts of higher plants have been accumulated over the past decade. These include the rapid degradation of unassembled subunits of protein complexes (Schmidt and Mishkind 1983; Feierabend et al. 1990), mistargeted proteins (Halperin and Adam 1996), photodamaged proteins (Aro et al. 1993) as well as developmentally regulated proteins (Reinbothe et al. 1995). Environmental factors such as light can also strongly affect the stability of chloroplast proteins (Aro et al. 1993; Lindahl et al. 1995; Reinbothe et al. 1995; Adamska et al. 1996). In isolated oat and barley chloroplasts, photooxidative conditions generated by varying the light intensity and O₂ concentration lead to an increased rate of degradation of several unidentified chloroplast proteins (Casano et al. 1990, 1994) as well as to a partial breakdown of the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Desimone et al. 1996). Obviously, proteolysis in chloroplasts plays a central role in housekeeping functions by removing superfluous or nonfunctional proteins, proteins which are damaged or stoichiometrically unbalanced subunits. In addition, massive protein degradation occurs within the chloroplast during leaf senescence, leading to decreased photosynthetic competence and the re-allocation of nitrogen and other mineral elements to growing organs (Feller and Fischer 1994; Smart 1994).

Although rapid protein degradation occurs in the chloroplast under defined conditions, knowledge of the identity and regulation of the relevant proteolytic enzymes is sparse. Specifically, proteolytic enzymes that regulate the senescence-associated degradation of abundant stromal enzymes such as Rubisco remain to be identified. A whole array of chloroplast peptidases

Abbreviations: CBB = Coomassie Brilliant Blue R-250; GOG-AT = glutamate synthase; GS = glutamine synthetase; LHCI = light-harvesting chlorophyll *a/b*-binding protein of PSII; LS = large subunit of Rubisco; PRK = phosphoribulokinase; SS = small subunit of Rubisco

Correspondence to: U. Feller; E-mail: urs.feller@pfp.unibe.ch;
 Fax: 41(31) 332 20 59

and proteases have been described, some of which require ATP (for review, see Adam 1996). Functional homologues of the bacterial, ATP-dependent Clp protease system have been identified in the stroma from several plant species (Shanklin et al. 1995). However, constitutive expression of Clp suggests that it is associated with general functions rather than in the direct regulation of senescence (Shanklin et al. 1995; Crafts-Brandner et al. 1996). Recent reports indicate that other homologues of bacterial proteases exist in chloroplasts of higher plants. Firstly, a protein similar to the cyanobacterial protease PrcA, a Ca^{2+} -stimulated but ATP-independent enzyme, has been detected in the stroma of higher-plant plastids (Ostersetzer et al. 1996). Secondly, a protein homologous to the ATP-dependent metalloprotease FtsH from *Escherichia coli* has been identified in the thylakoid membranes of chloroplasts (Lindahl et al. 1996). The expression of both proteins is regulated by light but their physiological function has not been determined. Recently a zinc-containing, Mg^{2+} -dependent stromal protease, EPI, has been partially purified from pea chloroplasts (Bushnell et al. 1993). In vitro, EPI is able to degrade the large subunit of isolated Rubisco to a smaller polypeptide of 36 kDa, suggesting that this enzyme may be responsible for the start of Rubisco turnover observed during senescence (Bushnell et al. 1993). Whether EPI or any of the aforementioned activities can actually use stromal proteins as substrates in vivo is largely unknown. Furthermore, links between proteases and chloroplast-specific protein degradation during senescence have not yet been established.

Intact chloroplasts isolated from mature pea leaves represent a good experimental system for studying proteolysis since degradation of nuclear-encoded proteins can be analyzed without the interference of concomitantly occurring synthesis. We previously showed that light strongly accelerated the degradation of several stromal enzymes involved in carbon and nitrogen metabolisms in intact chloroplasts while the same proteins were stable in the dark over several hours (Mitsuhashi and Feller 1992; Mitsuhashi et al. 1992). In the present work, we report on another situation during which stromal proteins are slowly degraded in organello independent of illumination. The inhibitor sensitivity profile, as well as the absolute requirement for divalent cations, strongly suggests that one or several zinc-containing, metallo-type proteolytic activities are involved in the process. Moreover, experiments carried out with chloroplasts isolated from leaves induced to senesce by nitrogen starvation provide the first information on a possible link between a chloroplast proteolytic system and stromal protein degradation observed during senescence, thus making the activities involved a suitable target for future physiological and molecular studies.

Materials and methods

Plant culture. Seeds of *Pisum sativum* L. cv. Piccolo petit provençal (Wyss Samen-Pflanzen, Zuchwil, Solothurn, Switzerland) were germinated on paper towels in the dark for 4 d at 25 °C. The

seedlings were then transplanted into 250 mL beakers (three plants per beaker) and grown hydroponically in a full-strength complete nutrient solution (Hildbrand et al. 1994). Nitrogen starvation was initiated at 12 to 13 d after germination, at the time when the third leaf was at about 50% of its final length, by removing the cotyledons from the seedlings and by replacing the nutrient solution by a minus-N solution (Crafts-Brandner et al. 1996). For comparative purposes, cotyledons of control plants (plus N) were also removed. Nutrient solutions were routinely changed when the beakers were half-empty. Plants were grown using a 14-h photoperiod with a photon fluence rate of $130 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetically active radiation. Day and night temperatures were 25 °C and 21 °C, respectively.

Isolation and incubation of intact chloroplasts. Prior to chloroplast isolation, the plants were kept in darkness for 24 h in order to reduce the starch content of the organelles. Chloroplasts were isolated on Percoll steps (21–60–80%) from mature, healthy leaves at 18 to 19 d after germination as described by Mitsuhashi et al. (1992). When chloroplasts were isolated from control and N-stressed plants, only the leaves formed at the time of N-stress initiation were used for the isolation. After collecting the intact chloroplasts from the 80% surface, the remaining Percoll was removed by several washes in the grinding buffer (standard medium) containing 50 mM Hepes-NaOH (pH 6.8), 350 mM sorbitol, 2 mM $\text{Na}_2\text{-EDTA}$, 1 mM MgCl_2 , 1 mM MnCl_2 , 5 mM isoascorbic acid, 1 mM dithiothreitol (DTT), 1% (w/v) polyethylene glycol-4000 and 0.001% (w/v) spectinomycin (Sigma, Buchs, St. Gallen, Switzerland) or in buffers of the desired composition. Intact chloroplasts were finally resuspended to a concentration of $200 \mu\text{g chlorophyll} \cdot \text{mL}^{-1}$ and aliquots of 200 to 500 μL were incubated at $25 \text{ °C} \pm 0.5 \text{ °C}$ in the dark or in the light ($45 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Following incubation, a second isolation of intact chloroplasts was carried out on Percoll steps (21 and 80%) as described by Mitsuhashi et al. (1992) to prevent contamination by lysed chloroplasts. The integrity of the organelles was routinely judged by phase-contrast microscopy.

Sodium dodecyl sulfate-PAGE, immunoblotting and densitometric analyses. Gel electrophoresis was carried out according to Laemmli (1970) using 0.75- or 1.5-mm-thick slab gels (12%). Collected samples (intact chloroplasts) were diluted with sample buffer [196 mM Tris-HCl (pH 6.8), 6.3% (w/v) SDS, 16% (v/v) β -mercaptoethanol, 32% (v/v) glycerol, 0.02% (w/v) bromophenol blue] in a ratio 2:1 (v/v) and heated at 95 °C for 5 min. Each lane was loaded with equal amounts of chlorophyll (1 μg). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (CBB) for detection of the protein pattern or blotted onto nitrocellulose membranes (0.45 μm ; Bio-Rad, Glattbrugg, Zürich, Switzerland) for the immunodetection of specific stromal proteins. Immunoblotting was performed as described by Mitsuhashi and Feller (1992) except that the nitrocellulose membranes were exposed overnight at 4 °C to the primary antibodies. The primary antibodies were kindly provided by G. Ochs and A. Wild (Johannes-Gutenberg-Universität, Mainz, Germany) against glutamine synthetase (GS), by S.J. Crafts-Brandner (Western Cotton Research Laboratory, USDA/ARS, Phoenix, Ariz. USA) against phosphoribulokinase (PRK), by R.M. Wallsgrove (IACR-Rothamsted, Harpenden, UK) against glutamate synthase (GOGAT), and by M. Mulligan (University of California, Irvine, USA) and R. Houtz (University of Kentucky, Lexington, USA) against a synthetic version of the first 25 amino acids from the N-terminal region of the large subunit of spinach Rubisco. Protein abundance was estimated by scanning the gels and immunoblots using a GS300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif., USA).

Chlorophyll and soluble protein. Leaves were homogenized in 20 mM sodium phosphate buffer (pH 7.5) containing 1% (w/v) polyvinylpyrrolidone and 0.1% (v/v) β -mercaptoethanol with a Polytron mixer (Kinematica, Littau, Luzern, Switzerland). The

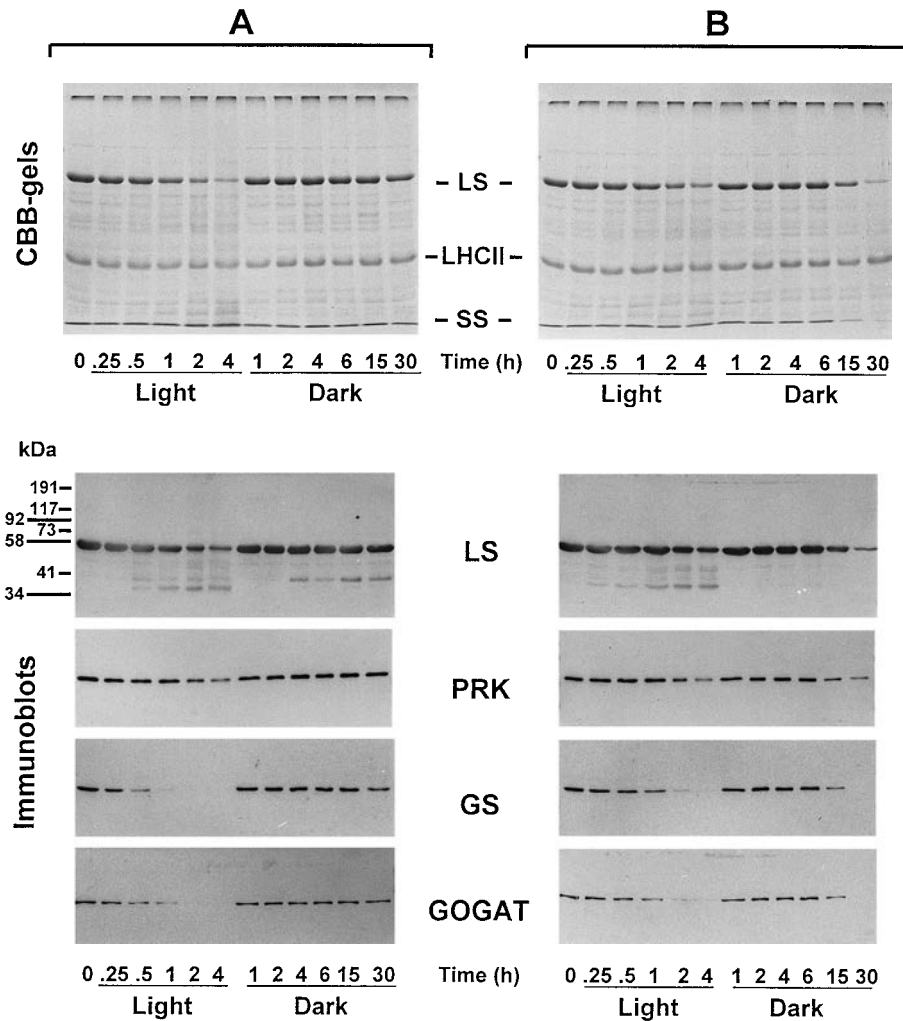


Fig. 1A,B. Proteolysis in intact *Pisum sativum* chloroplasts incubated in the light or in darkness. Isolated chloroplasts ($200 \mu\text{g chlorophyll} \cdot \text{mL}^{-1}$) were incubated in the light or in the dark in standard medium containing 1 mM DTT, 1 mM MgCl₂ and 2 mM EDTA (A) or in a modified medium lacking EDTA (B). Following incubation and re-isolation of the intact organelles, changes in the levels of several stromal proteins were visualized by Coomassie-staining (CBB) of the gels or by immunoblotting with specific antibodies

homogenate was filtered through Miracloth, and the filtrate (10–20 μL) or chloroplast suspension (5 to 10 μL) was mixed with 1 mL of 80% acetone, the chlorophyll quantified according to Strain et al. (1971). Soluble protein (supernatant of the Miracloth filtrate after centrifugation at 12 000 g for 10 min) was determined by the method of Bradford (1976) using horse IgG as standard.

Results

Light-stimulated and light-independent proteolysis in intact pea chloroplasts. We previously showed that illumination of intact chloroplasts isolated from mature pea leaves led to the rapid catabolism of several stromal proteins whereas the same proteins were stable in the dark over several hours of incubation (Mitsuhashi and Feller 1992; Mitsuhashi et al. 1992). The standard incubation medium used for such experiments contained, among other components, DTT (1 mM), MgCl₂ (1 mM) and EDTA (2 mM). Under such conditions and in the light, a large proportion of LS quickly disappeared concomitantly with the appearance of several degradation products in the molecular weight range 45–32 kDa (Fig. 1A). It should be noted that such products still contain the N-terminal part of LS since the antibody

used was produced against the N-terminal end of spinach LS. In contrast to LS, the small subunit of Rubisco (SS) was clearly less sensitive to light degradation (CBB-stained gels in Fig. 1). Other stromal proteins were also rapidly degraded following illumination of the chloroplasts. The levels of GS and GOGAT were markedly reduced after a 30-min incubation while PRK was clearly more stable. In the dark, proteins were stable for up to 30 h although a product of LS catabolism in the range of 37 kDa became visible after 4 h of incubation (Fig. 1A). In the absence of DTT from the incubation medium, light-stimulated proteolysis was markedly suppressed (Fig. 2).

In contrast to this rapid light-stimulated proteolysis, a more progressive light-independent protein degradation was observed when EDTA was omitted from the standard incubation medium (Fig. 1B). Lack of EDTA significantly decreased the stability of stromal proteins in dark-incubated chloroplasts and degradation of all investigated proteins was visible after 15 h of incubation. Unlike the rapid degradation observed in the light in the presence of DTT, no products of LS catabolism were observed throughout the incubation period. It should be pointed out that SS was also degraded under

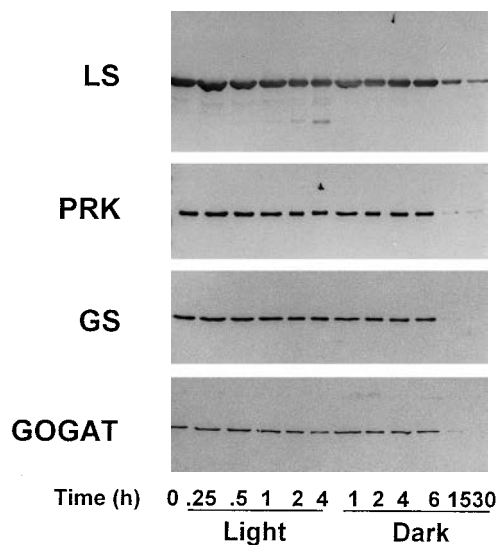


Fig. 2. Proteolysis in intact *Pisum sativum* chloroplasts incubated in the light or in the dark in the absence of DTT and EDTA. Isolated chloroplasts ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) were incubated in the light or in the dark in a medium containing no DTT and no EDTA. Changes in the levels of stromal proteins were visualized by immunoblotting after re-isolation of the intact chloroplasts. The same batch of chloroplasts as for Fig. 1 was used to allow a direct comparison of the results

these conditions (CBB-stained gel in Fig. 1B). The absence of EDTA from the incubation medium did not markedly alter the pattern of protein degradation observed in the light when DTT was present (Fig. 1A,B). On the other hand, proteolysis observed in the dark in the absence of EDTA was not stimulated by DTT; rather, the process was clearly delayed when 1 mM DTT was added to the medium (Figs. 1B, 2). Due to this inhibitory effect, DTT was omitted from the incubation medium for all subsequent experiments of light-independent proteolysis. The lack of formation of high-molecular-weight aggregates (immunoblots in Fig. 1) or of non-penetrating proteins on the top of the gel (CBB-stained gel in Fig. 1) indicated that the decreases in protein quantities in the light or in the dark were due to degradation within the plastids. Protein losses due to leakage into the medium could also be ruled out since only intact chloroplasts were analyzed following incubation. In contrast to stromal proteins, levels of the light-harvesting chlorophyll *a/b*-binding protein of PSII (LHCII) remained stable throughout the incubation (CBB-stained gel in Fig. 1), consistent with the lack of changes in chlorophyll abundance in our system (Mitsuhashi and Feller 1992).

Taken together, these results demonstrated two situations in which stromal proteins were degraded in organello. In one case, proteolysis was strongly stimulated by light in the presence of DTT. In the other case, protein degradation occurred in the dark in the absence of EDTA and was further enhanced by removal of DTT from the incubation medium. Earlier work in our laboratory was focused on the effects of light on stromal protein stability (Stieger and Feller 1997a,b). In the

Table 1. Inhibition of light-independent proteolysis by metalloprotease inhibitors. Isolated pea chloroplasts ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) were incubated for 25 h in darkness in standard medium containing no DTT but supplemented with the inhibitors at the indicated concentrations. Following re-isolation of the intact organelles, CBB-stained gels were produced for densitometric analyses of LS abundance based on the intensity of staining. Data are presented as relative values with the data obtained for unin-cubated controls set at 100

Compound added	Concentration	Relative LS Abundance
None	—	27
EDTA	2 mM	97
1,10-phenanthroline	0.1 mM	73
ZnCl ₂	2 mM	87
KCl	4 mM	14

current work, emphasis was placed on the light-independent proteolysis in intact chloroplasts. In our subsequent investigations, we mainly utilized Rubisco as a model substrate for chloroplast proteases.

Properties of light-independent proteolysis in intact pea chloroplasts. The nearly complete inhibition of stromal protein degradation in the dark observed when EDTA was present in the incubation medium (Fig. 1A) strongly suggested that metalloprotease activities were involved in the process. This assumption was reinforced by using the strong metal-chelator 1,10-phenanthroline (*o*-phenanthroline) as a more specific inhibitor and diagnostic for metallo-enzymes (Barrett 1986). In the presence of 0.1 mM *o*-phenanthroline, LS degradation was also inhibited but somewhat less than in the presence of 2 mM EDTA (Table 1). However, a direct comparison of EDTA and *o*-phenanthroline is very difficult in this context because the two chelators differ not only in their metal-complexing activities but also in their membrane permeabilities. The *o*-phenanthroline concentration could not be increased in our system since chloroplasts rapidly burst at concentrations above 0.1 mM. The fact that these inhibition experiments were carried out in standard medium containing 1 mM MgCl₂ implied that *o*-phenanthroline inhibited the process even in the presence of excess Mg²⁺. Light-independent degradation of Rubisco was also strongly inhibited by millimolar levels of ZnCl₂ while the addition of KCl had no inhibitory effect (Table 1). It should be pointed out that these inhibition studies were complicated by the fact that degradation of stromal proteins could not be maintained following lysis of the chloroplasts. Thus, restricted penetration of the chloroplast envelope might greatly reduce the interaction between an inhibitor and its target protease, making negative results difficult to interpret.

Inhibition of stromal protein degradation by metal-chelators such as EDTA and *o*-phenanthroline suggested that divalent cations were essential for sustaining optimal rates of proteolysis under our conditions. After removing metal ions by preincubating the chloroplasts in the presence of 5 mM EDTA for 6 h, no degradation of LS could be observed when chloroplasts were further

Table 2. Metal ion requirements. Isolated pea chloroplasts ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) were incubated for 6 h in darkness in the presence of 5 mM EDTA (inhibition). Following re-isolation, intact organelles were washed and resuspended ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) in standard buffer containing no EDTA and no DTT but supplemented with chloride salts of different cations. These suspensions were further incubated in darkness for 25 h before intact chloroplasts were re-isolated. Gels stained with CBB were used for analysis of LS abundance based on the intensity of staining. Data are presented as relative values with the data obtained just after the first 6 h of incubation set at 100

Salt added	Concentration	Relative LS Abundance
None	–	98
KCl	8 mM	94
MgCl ₂	4 mM	13
CaCl ₂	4 mM	19
MnCl ₂	4 mM	29
ZnCl ₂	20 μM	57

incubated for 25 h in darkness without EDTA and divalent cations in the medium (Table 2). The degradative activity could be fully restored by adding back millimolar levels of Mg^{2+} , Mn^{2+} or Ca^{2+} , and partial recovery of activity occurred when micromolar amounts of Zn^{2+} were added (Table 2).

When both DTT and EDTA were absent from the incubation medium, stromal proteins were relatively stable in the light for up to 4 h while clear decreases in their levels could be observed after 15 h incubation in the dark (Fig. 2). However, when chloroplasts were incubated for extended periods (15 h resp. 25 h) in the light under such conditions, degradation of LS also occurred and seemed to be even more pronounced than in the dark (Fig. 3). The presence of 2 mM EDTA in the incubation medium completely inhibited proteolysis in the dark but not in the light (Fig. 3).

Possible relevance of the system for protein degradation during leaf senescence. To address the question of whether light-independent proteolysis in isolated chloroplasts

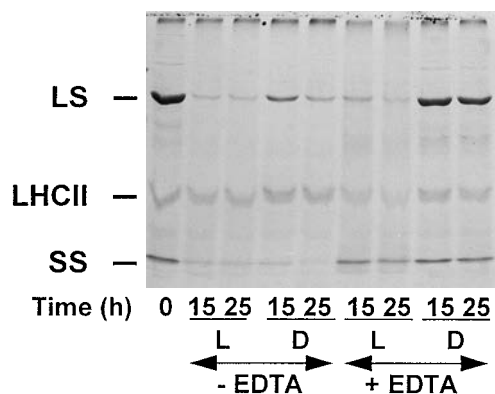


Fig. 3. Effect of EDTA on the degradation of LS in intact *Pisum sativum* chloroplasts incubated in the light (L) or in the dark (D) in the absence of DTT. Isolated chloroplasts ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) were incubated in the presence or absence of 2 mM EDTA. Following re-isolation, the levels of Rubisco protein in the intact organelles were visualized on CBB-stained gels

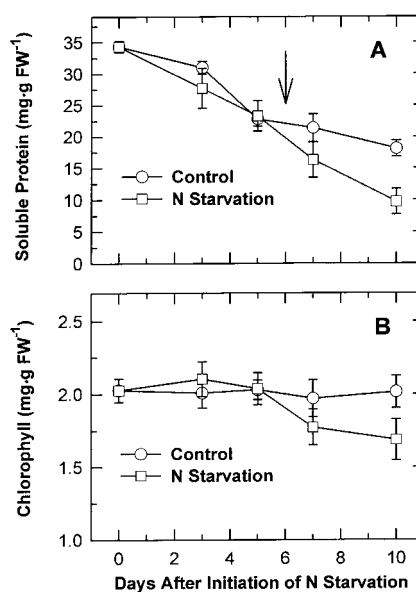


Fig. 4A,B. Abundances of soluble protein (A) and chlorophyll (B) per unit leaf fresh weight during natural aging (Control) and N-stress-induced senescence (N Starvation) of the first three leaves of *Pisum sativum*. Nitrogen starvation was initiated at 13 d after germination. Data are presented as means \pm SE of three replicates. The arrow indicates the time point at which chloroplasts were isolated during N-stress-induced senescence

roplasts might be indicative of chloroplast protein degradation occurring during senescence, we compared the process in chloroplasts isolated from control leaves with leaves that had been induced to senesce by N deficiency. Withdrawal of N from the nutrient solution represents a convenient way of inducing leaf senescence as indicated by the decline in soluble protein and total RNA abundances, and the coordinate loss of stromal proteins such as Rubisco and PRK (Crafts-Brandner et al. 1996). Figure 4 shows the time-course for the changes in soluble protein and chlorophyll abundances, two indicators for the progression of senescence, in the first three leaves of control and N-stressed plants. For both parameters, values started to deviate from the control 7 d after initiation of N deficiency (Fig. 4A,B). For further studies, chloroplasts were routinely isolated at day 6 after transfer of the plants to an N-free medium (see arrow in Fig. 4A), a time just before N starvation caused major effects on the content of soluble protein. This time point was considered as the putative “onset” of N-stress-induced senescence. It should be pointed out that leaves of control plants also underwent natural aging as indicated by the consistent decrease in soluble protein abundance during culturing (Fig. 4A) and had lost about 36% of their soluble protein at the time of chloroplast isolation. Upon incubation of chloroplasts isolated from control leaves under conditions stimulating light-independent proteolysis (–DTT, –EDTA), LS and PRK abundances were not markedly affected until 15 h of incubation, and about 30% of the initial amounts were still present after 25 h (Fig. 5A,B). In chloroplasts isolated from

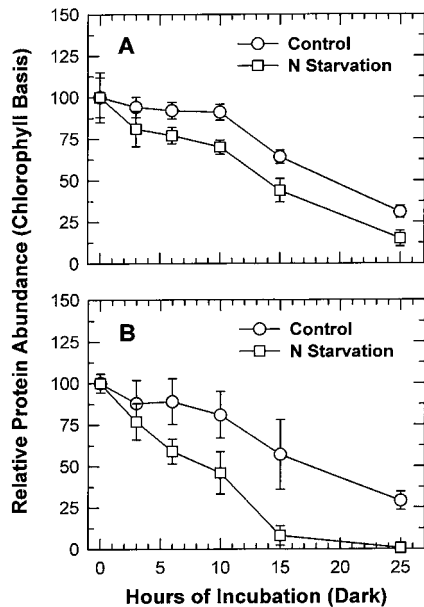


Fig. 5A,B. Time-course for the changes in Rubisco (A, based on LS) and PRK (B) abundances in chloroplasts isolated from control and N-starved *Pisum sativum* leaves, following incubation in the dark in the absence of DTT and EDTA. Chloroplasts were isolated at day 6 after initiation of N starvation (see arrow in Fig. 4A). Data are presented as relative values \pm SE of four independent incubations, with the data obtained for unincubated chloroplasts set at 100

N-starved leaves however, the abundances of both stromal enzymes declined more rapidly. After 10 h of incubation, levels of LS and PRK were already down to 70% and 42%, respectively, of their initial value, and no detectable amount of PRK was left after 25 h (Fig. 5A,B). These results indicated that the differences expected to occur *in vivo* at the level of Rubisco and PRK abundances between control and N-starved leaves (Crafts-Brandner et al. 1996) could be experimentally followed *in vitro* in intact, isolated chloroplasts. It is noteworthy that such experiments were done in darkness to avoid any interfering effects of light on stromal protein stability. The total chlorophyll content in chloroplast suspensions derived from control leaves or N-starved leaves remained constant throughout the incubation period under the conditions used (data not shown). Complete blockage of PRK degradation by EDTA in chloroplasts from both control and N-starved leaves (Fig. 6) clearly indicated that protein degradation in these two plastid populations depended on the same, metal-requiring proteolytic system.

Since "artificially-induced" and natural senescence may differ considerably in their expression and may not always be related to each other (Smart 1994), similar experiments were carried out with chloroplasts isolated from naturally senescent leaves and non-senescent leaves of the same plant. The results obtained were similar but the nutrient-deprived system was preferred because uniform senescence could be induced in a more reproducible and controlled way.

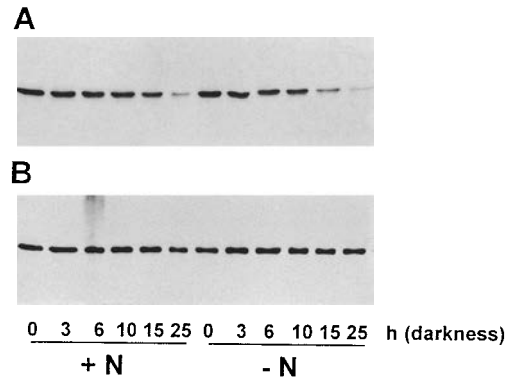


Fig. 6A,B. Effect of EDTA on the degradation of PRK in chloroplasts isolated from control (+N) or N-starved (-N) leaves of *Pisum sativum* following incubation in the dark. Isolated chloroplasts ($200 \mu\text{g}$ chlorophyll $\cdot \text{mL}^{-1}$) were incubated in the absence (A) or presence (B) of 2 mM EDTA. Changes in the levels of PRK were visualized by immunoblotting after re-isolation of the intact organelles

Discussion

It has been previously demonstrated that intact-isolated-chloroplasts are capable of rapidly degrading, in a light-stimulated manner, several stromal proteins involved in photosynthetic carbon and nitrogen metabolisms (Mitsuhashi and Feller 1992; Mitsuhashi et al. 1992). This process is most likely not limited by the availability of ATP (Stieger and Feller 1997a) but is accelerated by the production of activated oxygen species (Stieger and Feller 1997b). Similarly, other authors have reported increased rates of proteolysis in intact chloroplasts under oxidising conditions (Casano et al. 1990, 1994; Desimone et al. 1996), suggesting that oxidative modification of the substrate proteins might represent an important component of the proteolytic system. The strong stimulation of light-induced proteolysis by DTT observed in our experiments (Figs. 1, 2) also supported the view that free radicals participated in the process. This thiol reagent has been shown to strongly inhibit violaxanthin de-epoxidase and ascorbate peroxidase, two central enzymes of the radical detoxifying mechanisms of the chloroplast (Neubauer 1993). Although these experiments on light-stimulated proteolysis demonstrated that isolated chloroplasts have the capacity for degrading stromal proteins, it can be argued that these results do not reflect the processes that occur during leaf senescence. In particular, the participation of free radicals in chloroplast protein degradation during senescence and under various stress conditions still remains uncertain. In the present work, we focused on another situation during which native, stromal proteins were slowly degraded in organello in a light-independent but EDTA-sensitive manner.

Several characteristic features, including inhibition by EDTA, slower degradation rates, minimal accumulation of visible LS degradation products, and lack of stimulation by DTT, differentiated proteolysis in the dark from light-stimulated proteolysis (Figs. 1, 2). Since we utilized an intact system made up of a complex mixture

of different proteolytic activities, we can not be certain about the sequence of events leading to proteolysis of the different stromal proteins investigated. In general, it is thought that proteins destined for degradation are first hydrolysed by endoproteases into smaller peptides that can be further degraded to free amino acids by exopeptidases (Herbers et al. 1994; Callis 1995). We can therefore assume that, in our system, an endoproteolytic activity participated in the initial, rate-limiting cleavage of stromal proteins. The dependency on divalent cations (Table 2) and the inhibitor-sensitivity profile (Table 1) strongly suggested that a metalloprotease was involved. Additionally, inhibition by 1,10-phenanthroline in the presence of excess Mg^{2+} (Table 1) and the finding that micromolar levels of zinc ions could partially restore the activity after the system had been depleted of divalent cations (Table 2) further suggested that the enzyme might be a zinc-containing protease. Other proteases in this group, although containing catalytic amounts of zinc, are inhibited by millimolar concentrations of Zn^{2+} and stabilized by Ca^{2+} , and many of them are inhibited by DTT (Barrett 1986). The activity operating in our system meets most of these criteria. It is open to debate whether the inhibition of light-independent proteolysis by DTT (Figs. 1B, 2) is caused indirectly (e.g. interaction with metal ions) or directly by influencing a peptide hydrolase (e.g. changes in the redox state). Metallo-endopeptidase activities have been identified in plant plastids (Liu and Jagendorf 1986; Abad et al. 1989; Musgrove et al. 1989; Bushnell et al. 1993), and recently a homologue of a bacterial metalloprotease has been detected in the stromal-exposed regions of the thylakoid membranes (Lindahl et al. 1996). Although such enzymes might all use soluble proteins as substrates, the pea stromal metalloprotease EP1 characterized by Liu and Jagendorf (1986) and further purified by Bushnell et al. (1993) appears to be a prime candidate. The enzyme EP1 exhibits most of the properties we found associated with light-independent proteolysis in intact pea chloroplasts. Additionally, EP1 was shown to degrade the large, but not the small, subunit of purified Rubisco in vitro (Bushnell et al. 1993). Under our conditions, which would be favorable for EP1 activity, we found degradation of LS and SS, and several other stromal proteins, (Fig. 1B), indicating that these proteins might be physiological substrates for such an activity in organello. Whether proteolysis of the individual enzymes resulted from one single activity rather than from the interplay of several, closely related proteases remains, however, unclear.

It must be physiologically important to restrict, in vivo, proteolytic activities capable of degrading key enzymes such as Rubisco. Degradation of Rubisco in intact chloroplasts incubated in the absence of EDTA and DTT occurred both in the light and in the dark (Fig. 3), indicating that the stromal levels of Mg^{2+} or other essential divalent cations in the dark were sufficient for sustaining the activity and that light did not inhibit the process. Upon illumination, proteolysis of LS was even more pronounced and was no longer inhibited by EDTA. In this context, light-induced increases in the

stromal pH (Heldt et al. 1973) or in the stromal Mg^{2+} concentration (Portis and Heldt 1976) may become relevant in intact leaves. However, it is not ruled out that alternative control mechanisms also operate in the light, e.g. oxidative modification of proteins that increases their susceptibility to degradation or the availability of ATP. It remains a challenge for the future to determine whether such a proteolytic process capable of degrading native, stromal proteins in organello may actually be regulated by the in vivo Mg^{2+} or ATP levels, and may preferentially recognize oxidatively modified proteins.

It may be at first questionable to observe that chloroplasts isolated from mature, non-senescent leaves can degrade native, stromal proteins at relatively high rates upon incubation in the dark. The progression of the senescence process can vary greatly depending on species, response to source-sink interactions, environmental factors and endogenous cytokinin synthesis (Feller and Fischer 1994; Smart 1994; Gan and Amasino 1995). However, the overriding observations suggest that the leaves of annual plants become predisposed to senesce near the time of full leaf expansion, after which the abundances of several stromal enzymes involved in carbon assimilation begin to decline (Crafts-Brandner et al. 1996). Thus, it can be inferred that, after full leaf expansion, net degradation of these enzymes occurs in vivo. The light-independent proteolysis we observed in vitro in intact, isolated chloroplasts might therefore be representative of protein degradation occurring in vivo. This assumption was supported by the observation that degradation of Rubisco and PRK was substantially enhanced in chloroplasts isolated from leaves of N-stressed compared to control plants. Thus, inducing senescence in vivo did directly affect the degradation rates of stromal proteins in vitro under our conditions, suggesting that such a process might be analogous to proteolysis that occurs in intact leaves during senescence. However, the crucial question as to what causes stromal proteins such as Rubisco to be degraded during senescence remains unanswered. In particular, to what extent protein degradation is controlled at the level of the protease activities rather than by modification of the substrate proteins that might destabilize them can not be addressed by the present results. It appears likely that, at least when it comes to unassembled or damaged polypeptides, degradation may be mediated by pre-existing proteases (Adam 1996). Whether during senescence individual stromal proteins are in some manner altered prior to degradation remains unclear. Our data, however, suggest that a metal-requiring proteolytic system is participating in the process. Further characterization of this system at the physiological, biochemical and molecular levels will provide a better understanding of its regulation in vivo and its importance during senescence-specific and stress-related proteolysis in the chloroplast.

The authors thank Dr. S.J. Crafts-Brandner for helpful discussions and constructive comments on this work, and for improving the English of the manuscript. We are also grateful to Dr. G. Ochs and Prof. A. Wild, Dr. S.J. Crafts-Brandner, Dr. R.M. Wallsgrave, and Drs. M. Mulligan and R. Houtz for their kind gifts of the primary

antibodies used for the immunological detection of stromal proteins. This work was supported by the Swiss National Science Foundation.

References

- Abad MS, Clark SE, Lamppa GK (1989) Properties of a chloroplast enzyme that cleaves the chlorophyll a/b binding protein precursor. *Plant Physiol* 90: 117–124
- Adam Z (1996) Protein stability and degradation in chloroplasts. *Plant Mol Biol* 32: 773–783
- Adamska I, Lindahl M, Roobol-Boza M, Andersson B (1996) Degradation of the light-stress protein is mediated by an ATP-independent, serine-type protease under low-light conditions. *Eur J Biochem* 236: 591–599
- Aro EM, Virgin I, Andersson B (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim Biophys Acta* 1143: 113–134
- Barrett AJ (1986) The classes of proteolytic enzymes. In: Dalling MJ (ed.) *Plant proteolytic enzymes*, vol 1. CRC Press, Boca Raton, Florida, pp 1–16
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* 72: 248–254
- Bushnell TP, Bushnell D, Jagendorf AT (1993) A purified zinc protease of pea chloroplasts, EP1, degrades the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* 103: 585–591
- Callis J (1995) Regulation of protein degradation. *Plant Cell* 7: 845–857
- Casano LM, Gomez LD, Trippi VS (1990) Oxygen- and light-induced proteolysis in isolated oat chloroplasts. *Plant Cell Physiol* 31: 377–382
- Casano LM, Lascano HR, Trippi VS (1994) Hydroxyl radicals and a thylakoid-bound endopeptidase are involved in light- and oxygen-induced proteolysis in oat chloroplasts. *Plant Cell Physiol* 35: 145–152
- Crafts-Brandner SJ, Klein RR, Klein P, Hölzer R, Feller U (1996) Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of Clp protease subunits during senescence of *Phaseolus vulgaris* (L.) leaves. *Planta* 200: 312–318
- Desimone M, Henke A, Wagner E (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. *Plant Physiol* 111: 789–796
- Feierabend J, Bergmann C, Otto S (1990) Comparison of properties of the proteolytic degradation of unassembled nuclear-encoded subunits of ribulose-1,5-bisphosphate carboxylase and of the coupling factor of photophosphorylation CF1. *Bot Acta* 103: 174–182
- Feller U, Fischer A (1994) Nitrogen metabolism in senescing leaves. *Crit Rev Plant Sci* 13: 241–273
- Gan S, Amasino R (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* 270: 1986–1988
- Halperin T, Adam Z (1996) Degradation of mistargeted OEE33 in the chloroplast stroma. *Plant Mol Biol* 30: 925–933
- Heldt HW, Werdan K, Milovancev M, Geller G (1973) Alkalinization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. *Biochim Biophys Acta* 314: 224–241
- Herbers K, Prat S, Willmitzer L (1994) Functional analysis of a leucine aminopeptidase from *Solanum tuberosum* L. *Planta* 194: 230–240
- Hildbrand M, Fischer A, Feller U (1994) Protein catabolism in bean leaf discs: accumulation of a soluble fragment of ribulose-1,5-bisphosphate carboxylase/oxygenase under oxygen deficiency. *J Exp Bot* 45: 1197–1204
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
- Lindahl M, Yang D-H, Andersson B (1995) Regulatory proteolysis of the major light-harvesting chlorophyll a/b protein of photosystem II by a light-induced membrane-associated enzymic system. *Eur J Biochem* 231: 503–509
- Lindahl M, Tabak S, Cseke L, Pichersky E, Andersson B, Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. *J Biol Chem* 271: 29329–29334
- Liu X-Q, Jagendorf AT (1986) Neutral peptidases in the stroma of pea chloroplasts. *Plant Physiol* 81: 603–608
- Mitsuhashi W, Feller U (1992) Effects of light and external solutes on the catabolism of nuclear-encoded stromal proteins in intact chloroplasts isolated from pea leaves. *Plant Physiol* 100: 2100–2105
- Mitsuhashi W, Crafts-Brandner SJ, Feller U (1992) Ribulose-1,5-bis-phosphate carboxylase/oxygenase degradation in isolated pea chloroplasts incubated in the light or in the dark. *J Plant Physiol* 139: 653–658
- Musgrove JE, Elderfield PD, Robinson C (1989) Endopeptidases in the stroma and thylakoids of pea chloroplasts. *Plant Physiol* 90: 1616–1621
- Neubauer C (1993) Multiple effects of dithiothreitol on nonphotochemical fluorescence quenching in intact chloroplasts. *Plant Physiol* 103: 575–583
- Ostersetzer O, Tabak S, Yarden O, Shapira R, Adam Z (1996) Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts. *Eur J Biochem* 236: 932–936
- Portis AR, Heldt HW (1976) Light-dependent changes of the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. *Biochim Biophys Acta* 449: 434–446
- Reinbothe C, Apel K, Reinbothe S (1995) A light-induced protease from barley plastids degrades NADPH:protochlorophyllide oxidoreductase complexed with chlorophyllide. *Mol Cell Biol* 15: 6206–6212
- Schmidt GW, Mishkind ML (1983) Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase small subunit in chloroplasts. *Proc Natl Acad Sci USA* 80: 2623–2636
- Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. *Plant Cell* 7: 1713–1722
- Smart CM (1994) Gene expression during leaf senescence. *New Phytol* 126: 419–448
- Stieger PA, Feller U (1997a) Requirements for the light-stimulated degradation of stromal proteins in isolated pea (*Pisum sativum* L.) chloroplasts. *J Exp Bot* 48: 1639–1645
- Stieger PA, Feller U (1997b) Degradation of stromal proteins in pea (*Pisum sativum* L.) chloroplasts under oxidising conditions. *J Plant Physiol* 151: 556–562
- Strain HH, Cope BT, Svec WA (1971) Analytical procedures for the identification, estimation and investigation of the chlorophylls. *Methods Enzymol* 23: 452–487