



Requirements for the light-stimulated degradation of stromal proteins in isolated pea (*Pisum sativum* L.) chloroplasts

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Abstract

Chloroplasts from 17-d-old pea leaves (*Pisum sativum* L.) were isolated to elucidate the requirements for the light-induced degradation of stromal proteins. The influence of electron transport through the thylakoids and the influence of ATP on protein degradation were investigated. When chloroplasts were incubated in the light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$), glutamine synthetase, the large subunit of ribulose-1,5-bisphosphate carboxylase and glutamate synthase were degraded, whereas phosphoribulokinase, ferredoxin-NADP⁺ reductase and the 33 kDa protein of photosystem II remained more stable. Major protein degradation was not observed over 240 min in darkness. The electron transport inhibitor dichlorophenylmethylurea reduced protein degradation in the light over several hours, whereas dibromothymoquinone was less effective. Inhibiting the production of ATP with tentoxin or by destroying the ΔpH with the ionophores valinomycin and nigericin had no effect or even a stimulating influence on protein degradation when chloroplasts were exposed to light. Furthermore, adding ATP to chloroplasts incubated in the dark had no effect on proteolysis. From these results it is concluded that the transport of electrons through the thylakoids or photooxidative processes associated with it (especially in presence of DTT), rather than the availability of ATP caused the acceleration of stromal protein degradation by light in isolated pea chloroplasts.

Key words: Isolated pea chloroplasts, light-induced protein degradation.

Introduction

Proteolysis is important for the development, function and senescence of plant organelles. Nuclear encoded proteins are proteolytically processed in chloroplasts after uptake across the envelope. In the plastids, newly synthesized immature or incorrectly assembled proteins are removed by proteolysis (Vierstra, 1993; Callis, 1995). Proteins damaged as a consequence of external stresses, such as an excess of light or low temperature, are also removed. For example, the D1 protein (32 kDa protein of PSII) is rapidly degraded in light, but not in darkness (Mattoo *et al.*, 1984; Aro *et al.*, 1993). The organelles of pea leaves contain about 80% of the total nitrogen of a leaf (Makino and Osmond, 1991). Thus, the degradation of proteins in the organelles and the translocation of nitrogen to growing organs is an important process during senescence. Several proteolytic systems in the stroma and associated with thylakoids are known. For example, an ATP-dependent protease has been found by several authors to be responsible for the removal of non-integrated subunits, newly synthesized proteins containing abnormal amino acids and prematurely terminated proteins (Liu and Jagendorf, 1984, 1985; Malek *et al.*, 1984). In addition an ATP-dependent protease is involved in the degradation of the 25 kDa protein of LHCII when plants are exposed to an excess of light (Lindahl *et al.*, 1995).

ATP-dependent proteolytic systems (La and Clp) have been described in detail in *E. coli* (Goldberg, 1992; Maurizi, 1992). The major function of these proteolytic systems is most likely to remove abnormal proteins. The plastids of higher plants contain ClpP and ClpC, homologues of the ClpP and ClpA of *E. coli* (Shanklin *et al.*, 1995). Genes encoding the subunit with ATPase activity

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(ClpC) have been identified in the nucleus of several plant species (Gottesmann *et al.*, 1990; Moore and Keegstra, 1993; Ko *et al.*, 1994; Shanklin *et al.*, 1995), whereas the subunit with proteolytic activity (ClpP) is encoded on the chloroplast DNA (Maurizi *et al.*, 1990; Shanklin *et al.*, 1995). Specific functions of the Clp system have not yet been reported for plants. In addition to ATP-dependent systems, a magnesium-stimulated, zinc-dependent protease (EP1) has been found in the stroma of pea chloroplasts and is able to degrade the large subunit (LS) of rubisco (EC 4.1.1.39) (Liu and Jagendorf, 1985, 1986; Bushnell *et al.*, 1993).

In isolated pea chloroplasts exposed to light, a rapid degradation of several stromal proteins has been observed, whereas in the dark these proteins remain stable for several hours (Mitsuhashi and Feller, 1992; Mitsuhashi *et al.*, 1992; Stieger and Feller, 1995). Other workers have shown that the production of activated oxygen species by increased light intensities, elevated oxygen concentrations, or by the addition of the herbicide methyl viologen caused a fragmentation of LS in isolated barley chloroplasts (Desimone *et al.*, 1996). Increased light and oxygen concentrations also accelerated protein degradation in isolated oat chloroplasts (Casano *et al.*, 1990; Casano and Trippi, 1992). Furthermore, oxidative stress initiated by the addition of CuSO₄ to *Spirodela* plants or to isolated wheat chloroplasts led to insolubilization, dimerization and degradation of LS (Mehta *et al.*, 1992). Similar effects were observed when *Euglena gracilis* was grown in nitrogen-deficient nutrient solution (Garcia-Ferris and Moreno, 1994).

The degradation of proteins in chloroplasts may be regulated by the abundance and activity of proteases or by the modification of substrate proteins (e.g. oxidation, phosphorylation, acetylation). The observed decrease of stromal proteins in the light might be dependent on ATP-consuming systems. On the other hand, activated oxygen species produced in illuminated chloroplasts might be responsible for an increased susceptibility of stromal proteins to proteolysis. In an attempt to distinguish between the requirement for ATP and effects of activated oxygen species, a series of specific inhibitors was used to block the electron transport or the formation of ATP and the effects on plastid protein breakdown were analysed.

Materials and methods

Plant material and isolation of chloroplasts

Pea plants (*Pisum sativum* L., cv. Piccolo Petit Provençal) were germinated on wet paper for 4 d and then grown for 14 d in hydroponic culture according to Hildbrand *et al.* (1994), with a photoperiod of 14 h (120 μmol photons m⁻² s⁻¹) at 25/21 °C day/night temperatures. Prior to chloroplast isolation the pea plants were kept for 24 h in the dark to reduce the starch content in the chloroplasts. Pea leaves were homogenized with a Polytron mixer (Kinematica, Luzern, Switzerland) in grinding

buffer (50 mM HEPES–NaOH, pH 6.8, 350 mM sorbitol, 2 mM Na₂-EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 5 mM isoascorbic acid, 1 mM dithiothreitol (DTT), 1% (w/v) polyethyleneglycol 4000, and 0.001% (w/v) spectinomycin), filtrated through two layers of Miracloth (Calbiochem, La Jolla, USA) and centrifuged for 1 min at 2000 g. The pellet was resuspended in grinding buffer and loaded on 40/80% (v/v) Percoll steps (Pharmacia, Uppsala, Sweden). To the 40% Percoll step bovine serum albumin (0.2%, w/v) was added. After centrifugation of the gradients for 12 min at 1800 g, the chloroplasts were collected from the 80% Percoll surface and resuspended in grinding buffer. The chloroplasts were then sedimentated by centrifugation for 3 min at 3000 g and resuspended in grinding buffer (200 μg chl ml⁻¹). The purity and intactness of chloroplasts isolated with this procedure were tested as reported previously by Mitsuhashi and Feller (1992). The chloroplast suspensions were incubated in low light (45 μmol m⁻² s⁻¹) or in darkness. Where indicated, effectors from stock solutions were added to the incubation medium. The ATP stock solution was neutralized with NaHCO₃. Chloroplast suspensions were kept in the dark at 25 °C for 10 min to allow the uptake of effectors into the chloroplasts before starting the experiments. After incubation, chloroplasts were reisolated on Percoll steps (21/80%) as described by Mitsuhashi and Feller (1992) to prevent contamination by lysed chloroplasts.

SDS-PAGE and immunoblotting

Collected samples were heated for 5 min with an equal volume of sample buffer (250 mM TRIS–HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.03% bromophenol blue). Gel electrophoresis was carried out according to Laemmli (1970) using 0.75 mm thick slab gels (12%). After electrophoresis, immunoblotting was carried out as described by Mitsuhashi and Feller (1992). Primary antibodies were kindly supplied by S Gepstein (Technion-Israel Institute of Technology, Haifa) against LS and against the 33 kDa protein of PSII; by G Ochs and A Wild (Johannes-Gutenberg-Universität, Mainz) against plastidial glutamine synthetase (GS, EC 6.3.1.2); by SJ Crafts-Brandner (Western Cotton Research Laboratory, USDA/ARS, Phoenix) against phosphoribulokinase (PRK, EC 2.7.1.19); by RM Wallsgrove (IACR-Rothamsted, Harpenden) against ferredoxin-dependent glutamate synthase (GOGAT, EC 1.4.7.1); and by S Ida (Research Institute for Food Science, Kyoto University, Kyoto) against ferredoxin-NADP⁺ reductase from rice leaves (FNR, EC 1.18.1.2). All the antibodies used were highly specific (Fig. 1).

Results

Protein degradation in isolated chloroplasts exposed to light was observed when DTT was present in the grinding buffer and in the incubation medium (Fig. 2A). This result was evident for LS when proteins were separated and visualized on a Coomassie Brilliant Blue gel and for GS on Western blots. Omitting DTT during chloroplast isolation and incubation stabilized these proteins over 180 min (Fig. 2A). The substitution of DTT with β-mercaptoethanol resulted in protein degradation when chloroplasts were exposed to light (Fig. 2B). However, in the dark the proteins remained stable in presence of β-mercaptoethanol. Thus, it appears likely that DTT can be replaced by other thiols. The following experiments

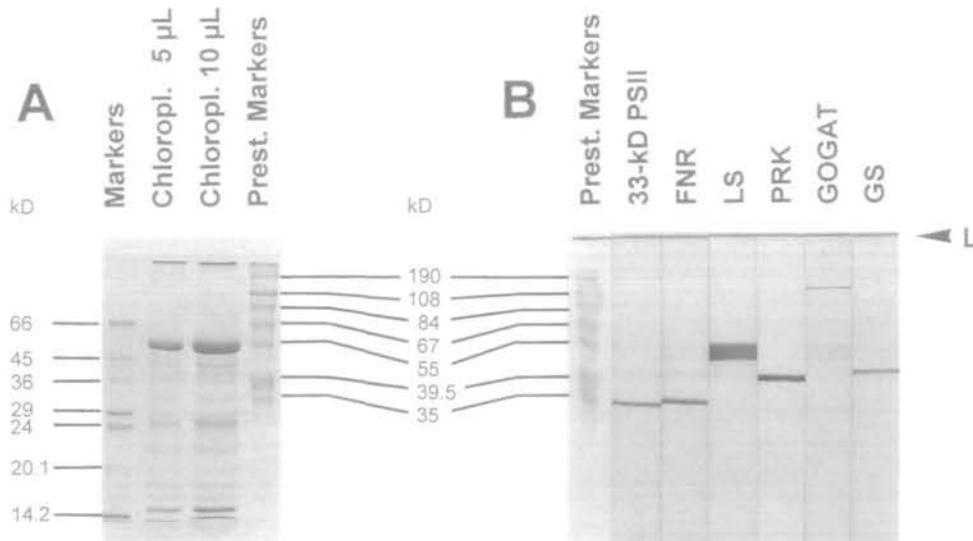


Fig. 1. Specificity of the antibodies used. The polyacrylamide gels (12%; 1 mm thick) for staining (A) and for immunoblots (B) were prepared simultaneously with a sample from freshly isolated chloroplasts (Chloropl.). Samples of 5 and 10 μL were loaded per lane (5 mm wide) for the stained gel. Markers (SIGMA Dalton Mark VII-L) and prestained markers (SIGMA SDS-7B) were loaded on separate lanes. A special comb with a small (3 mm wide) pocket for the prestained markers and with a large pocket (75 mm wide; loaded with 80 μL chloroplast sample) was used for immunoblotting. A line at the top of the nitrocellulose membrane (L) was drawn before cutting the blot into strips (8 mm wide). The various strips were decorated with the appropriate antibodies and processed according to the standard protocol for immunoblotting. After colour development, the strips were aligned in the original sequence for the photograph.

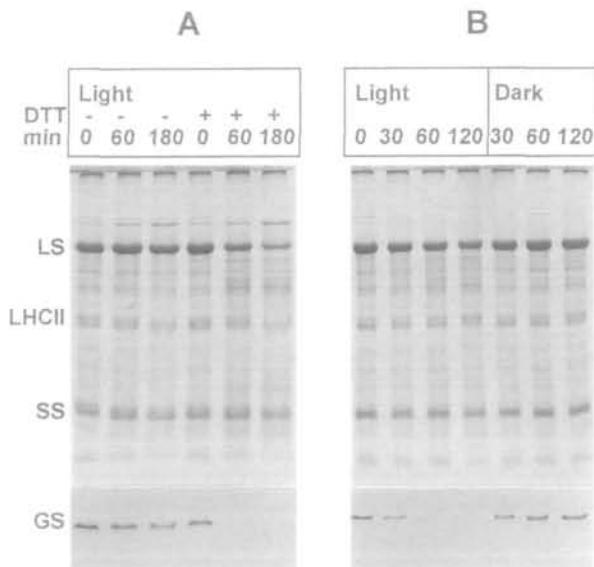


Fig. 2. Influence of thiols on protein degradation in intact chloroplasts. Chloroplast suspensions ($200 \mu\text{g chl ml}^{-1}$) were exposed to light in the presence (+) or absence (-) of 1 mM DTT (A), or in the presence of 1 mM β -mercaptoethanol (no DTT) (B). The thiols were added as indicated to the grinding buffer, washing solution and incubation medium. Intact chloroplasts were reisolated from samples taken at the times indicated and equal amounts of chl (1 μg per lane) were loaded on a 12% polyacrylamide gel. The large (LS) and small (SS) subunits of Rubisco and LHCII were detected on a stained gel, while GS was visualized by Western blotting.

were all carried out in presence of DTT during chloroplast isolation and incubation.

By adding dichlorophenyldimethylurea (DCMU) to the incubation medium the electron transport chain can

be inhibited at the Q_B site of PSII, whereas dibromothymoquinone (DBMIB) inhibits electron transport at the cyt b/f complex (Nicholls and Ferguson, 1992). These two inhibitors may also affect the production of ATP. Stromal proteins were degraded when chloroplasts were exposed to light (Fig. 3). Most susceptible to degradation in the light were GS, GOGAT and LS, whereas PRK decreased only slightly and FNR was stable. The thy-

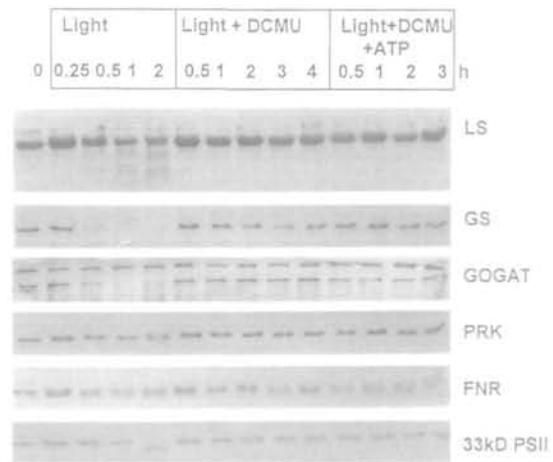


Fig. 3. Influence of DCMU and ATP on protein degradation in intact chloroplasts exposed to light. Chloroplast suspensions ($200 \mu\text{g chl ml}^{-1}$) were exposed to light and 5 μM DCMU or 5 mM ATP (final concentrations) were added to the incubation medium when indicated. Intact chloroplasts were reisolated from all samples and proteins were visualized by Western blotting. Equal amounts of chl (1 μg per lane for LS, GS, GOGAT, PRK, and the 33 kDa protein of PSII; 2 μg per lane for FNR) were loaded on a 12% polyacrylamide gel.

lakoid-bound 33 kDa protein of PSII did not show any instability at the light intensities used in this experiment. DCMU in the incubation medium reduced the degradation of stromal proteins drastically. It stabilized the light-susceptible proteins GS, GOGAT and LS over 4 h incubation. Adding DBMIB to the incubation medium was not as effective as DCMU (Fig. 4). The light-susceptible proteins GS, GOGAT and LS were only protected over a period of 60 min, afterwards their degradation was as fast as in chloroplasts exposed to light. Incubation of chloroplasts in darkness resulted in protein stability, regardless of the effectors added to the incubation medium (Figs 2, 4).

To differentiate between the influence of ATP and other effects of electron transport through the thylakoids, ATP was added to chloroplasts with DCMU (Fig. 3) or to chloroplasts incubated in the dark (data not shown). No stimulation of proteolysis by ATP was observed under such conditions. Tentoxin, an antagonist of the CF1 subunit of the ATPase, inhibits the production of ATP without directly influencing the electron transport through PSII and PSI (Avni *et al.*, 1992). Similarly, the ionophores nigericin and valinomycin abolish the proton gradient between the stroma and the lumen and, as a consequence, inhibit the production of ATP, but not the electron flow through the thylakoids (Nicholls and Ferguson, 1992). Stromal proteins, especially GS, GOGAT and LS, were degraded when chloroplasts were exposed to light, regardless of the addition of tentoxin to the incubation medium

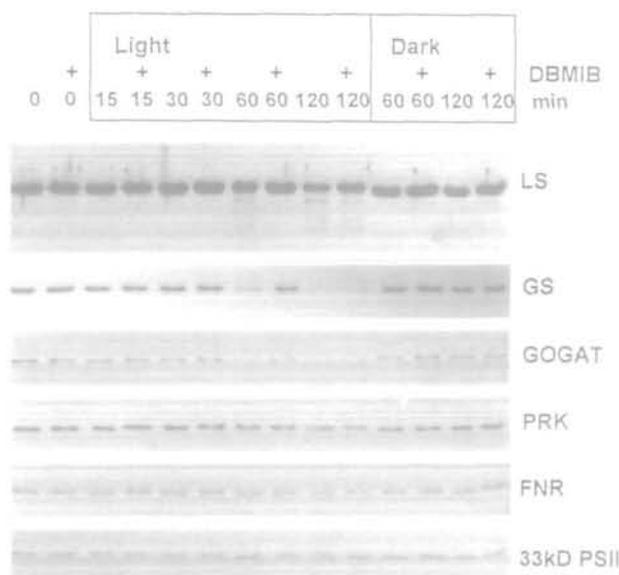


Fig. 4. Influence of DBMIB on protein degradation in intact chloroplasts. Chloroplast suspensions ($200 \mu\text{g chl ml}^{-1}$) were exposed to light or darkness and $5 \mu\text{M}$ DBMIB (final concentration) was added to the incubation medium when indicated (+). Intact chloroplasts were reisolated from all samples and proteins were visualized by Western blotting. Equal amounts of chl ($1 \mu\text{g}$ per lane for LS, GS, GOGAT, PRK, and the 33 kDa protein of PSII; $2 \mu\text{g}$ per lane for FNR) were loaded on a 12% polyacrylamide gel.

(Fig. 5). In the dark, proteins remained stable in the presence or in the absence of tentoxin. Once again, the proteins PRK, FNR and the 33 kDa protein of PSII were quite stable in chloroplasts exposed to light. The ionophores nigericin and valinomycin had stimulating effects on protein degradation in chloroplasts exposed to light (Fig. 6). Degradation products of LS became visible after 15 min and the amount of LS decreased faster than in controls without the inhibitor. Similar observations were made for GS, GOGAT and even PRK. The thylakoid-bound 33 kDa protein of PSII was stable during the incubation period. In the dark, all proteins remained quite stable and the ionophores had no effect.

Discussion

The degradation of the stromal proteins GS, GOGAT, and LS in illuminated pea chloroplasts was markedly reduced by the inhibition of electron transport with DCMU. The addition of ATP to such chloroplast suspensions or to chloroplasts incubated in darkness did not accelerate protein degradation. Furthermore, reduction of ATP production in isolated chloroplasts in the light with tentoxin, an antagonist of the CF1-subunit of the ATPase, did not inhibit the degradation of stromal proteins in the light. Destroying the proton gradient between the lumen and the stroma with ionophores even had a stimulating effect on protein degradation in the stroma.

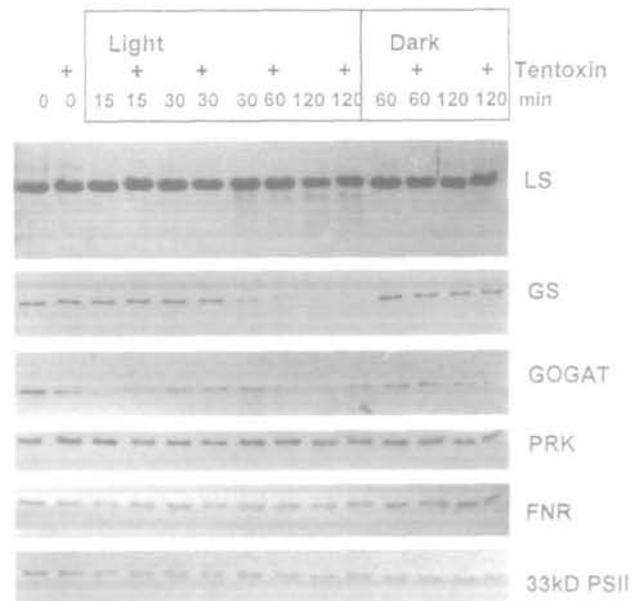


Fig. 5. Influence of tentoxin on protein degradation in intact chloroplasts. Chloroplast suspensions ($200 \mu\text{g chl ml}^{-1}$) were exposed to light or darkness and $5 \mu\text{M}$ tentoxin (final concentration) was added to the incubation medium when indicated (+). Intact chloroplasts were reisolated from all samples and proteins were visualized by Western blotting. Equal amounts of chl ($1 \mu\text{g}$ per lane for LS, GS, GOGAT, PRK, and the 33 kDa protein of PSII; $2 \mu\text{g}$ per lane for FNR) were loaded on a 12% polyacrylamide gel.

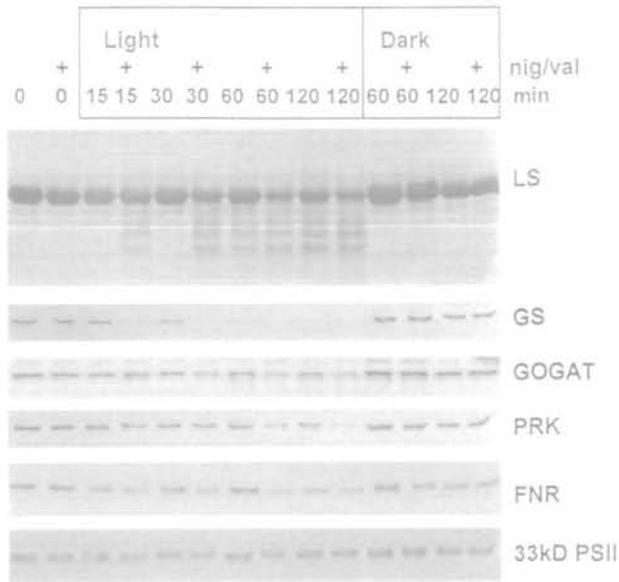


Fig. 6. Influence of the ionophores nigericin and valinomycin on protein degradation in intact chloroplasts. Chloroplast suspensions ($200 \mu\text{g chl ml}^{-1}$) were exposed to light or darkness and a combination of $5 \mu\text{M}$ nigericin, $5 \mu\text{M}$ valinomycin and 1 mM KCl (final concentrations) was added to the incubation medium when indicated (+). Intact chloroplasts were reisolated from all samples and proteins were visualized by Western blotting. Equal amounts of chl ($1 \mu\text{g}$ per lane for LS, GS, GOGAT, PRK, and the 33 kDa protein of PSII; $2 \mu\text{g}$ per lane for FNR) were loaded on a 12% polyacrylamide gel.

From these results, it was concluded that the availability of ATP is not crucial for the initial steps of GS, GOGAT and LS degradation.

Comparing the effects of the two electron transport inhibitors DCMU and DBMIB, it was noticed that DCMU was much more effective than DBMIB. DCMU binds to the Q_B -binding site of PSII, whereas DBMIB interacts with the cytochrome *b/f* complex. Elstner and Frommeyer (1978) measured no production of O_2^- and H_2O_2 when DCMU was added to isolated thylakoids, whereas the addition of DBMIB resulted in the formation of H_2O_2 . In the latter case, electrons are transferred from Q_A to Q_B forming the protonated plastoquinol from which electrons may be transferred to O_2 (Kyle, 1987).

The ATPase inhibitor tentoxin binds to the β -subunit of CF1 (Avni *et al.*, 1992) and has no major effects on the electron transport chain in the thylakoids (Bulychev and Dahse, 1984; Dahse *et al.*, 1986). Therefore, the amount of activated oxygen species produced would be very similar in tentoxin-treated and untreated chloroplasts exposed to light. In contrast, the ionophores nigericin and valinomycin in the presence of K^+ ions destroy the proton gradient and the ΔpH across the thylakoids, without reducing the flow of electrons through the membrane. The ΔpH is an important regulatory component in the dissipation of excess energy via the xanthophyll cycle, the xanthophyll cycle-dependent energy quenching being inhibited at a low ΔpH (Gilmore *et al.*, 1995).

Furthermore, DTT and β -mercaptoethanol strongly inhibit violaxanthin de-epoxidase and ascorbate peroxidase, two central enzymes of the radical detoxifying mechanisms known in chloroplasts (Yamamoto and Kamite, 1972; Chen and Asada, 1992; Neubauer, 1993). DTT in the presence of Fe^{3+} and O_2 might give rise to thiyl or oxygenated sulphur radicals (Netto and Stadtman, 1996). The effects of DTT causing an accumulation of radicals by increasing their formation or by inhibiting their detoxification could explain the improved stability of stromal enzymes in these experiments when chloroplasts were incubated in the light in the absence of DTT.

In previous work (Mitsuhashi *et al.*, 1992), LS fragments with molecular weights of 45, 42, 37, and 32 kDa were described. Recently, similar fragments were also detected when barley chloroplasts were exposed to high doses of oxygen and light or to the herbicide methyl viologen (Desimone *et al.*, 1996). These fragments are in the range between 48 and 36 kDa , the 36 kDa being the most abundant. Such polypeptides may accumulate under conditions which favour the production of free radicals. In *E. coli*, GS is known to be very susceptible to radical attack. The protein is first oxidized to a catalytically inactive form and then this form is quickly degraded by intracellular proteases (Levine *et al.*, 1981). In this experimental system, GS was the most labile of the stromal proteins investigated, its degradation being very fast in the light. Nevertheless, it remained stable over several hours when DTT was removed from the incubation medium, when DCMU was added or when chloroplasts were incubated in darkness. On the other hand, PRK, FNR and the 33 kDa protein of PSII were quite stable in chloroplasts exposed to light. The 33 kDa protein is peripherally bound to the oxygen-evolving system on the luminal side of the thylakoid membrane and functions as the Mn-stabilizing protein (Yamamoto, 1988). In addition, it stabilizes the CP43 protein under photoinhibitory conditions (Yamamoto and Akasaka, 1995). In bean leaves it remains stable until late in senescence (Roberts *et al.*, 1987). Thus, it was not surprising that no decrease of the 33 kDa protein was observed in this work. Proteins with a metal-binding site can be oxidized in a site-specific manner by reaction of Fe(II) with H_2O_2 forming hydroxyl radicals (Stadtman, 1992). In plants, Rubisco and GS possess a cation binding site near the catalytic centre which normally binds Mg^{2+} but which can also bind Fe^{2+} .

In this work the light-stimulated degradation of stromal proteins occurred only when the electron transport chain was functioning. The inhibition of ATP production did not influence this degradation process. From these results, it was concluded that the modification of stromal proteins by activated oxygen species rather than the stimulation of an ATP-dependent protease is the first step leading to the degradation of stromal enzymes. The three-

dimensional structure of the proteins may be relevant for their susceptibility to radical attack. The characterization and purification of proteases digesting such modified proteins will be a task for future work.

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