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Communication

Effects of Light and External Solutes on the Catabolism of Nuclear-Encoded Stromal Proteins in Intact Chloroplasts Isolated from Pea Leaves[1]

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ABSTRACT

The catabolism of nuclear-encoded stromal proteins was investigated in intact chloroplasts isolated mechanically from pea (*Pisum sativum*) leaves. Glutamine synthetase, phosphoribulokinase, and nitrite reductase (quantified by immunoblotting) were more rapidly degraded in the light than in the dark. Furthermore, the degradation rates depended on exogenously supplied metabolites. For example, 2-oxoglutarate accelerated the catabolism of all three enzymes in chloroplasts incubated in the light, whereas oxaloacetate stabilized glutamine synthetase and at the same time destabilized the other two enzymes.

Plastid-localized proteins turn over continually in mature leaves with net degradation occurring during senescence (21). Several endopeptidases and exopeptidases have been identified and characterized in chloroplasts from various plant species (8, 10–12, 15, 24). In pea (*Pisum sativum*), endopeptidases have been detected in the stroma (11, 15) and thylakoid membrane (15) fractions, and aminopeptidases were located in the stroma (11). However, the function of the various peptide hydrolases in the catabolism of chloroplast proteins and the regulation of proteolysis in plastids are not yet clear. Nuclear-encoded stromal proteins, such as PRK[2], NiR, and plastid GS are synthesized in the cytosol and then imported into the chloroplast (7, 17). Therefore, any net change in the quantity of these proteins within isolated intact chloroplasts can only depend on catabolism occurring within the chloroplasts. In vitro protein stability is affected by metabolites, but it is still uncertain to what extent such interactions might be relevant in vivo (20). Solutes are easily exchanged across the chloroplast envelope via specific translocator proteins (5). The concentrations of Calvin cycle intermediates and other metabolites depend on a number of external and internal factors (1). The catabolism of Rubisco and the accumulation of breakdown products depend on illumination and on the composition of the incubation medium (14). In this paper, we have analyzed the effect that various external metabolites and illumination have on the degradation of three nuclear-encoded proteins within intact isolated pea chloroplasts.

MATERIALS AND METHODS

Isolation and Incubation of Chloroplasts

Pea (*Pisum sativum* L.) seeds were germinated on wet paper in the dark at 25°C for 3 d. The seedlings were transferred to quartz sand and grown for 3 d with a photoperiod of 14 h (120 $\mu E\ m^{-2}\ s^{-1}$). These plants were then transferred to hydroponic culture with low nitrate solution according to Thomas et al. (22), except 15 $\mu g/L$ $Ni(NO_3)_2 \cdot 6H_2O$ was added. Plants (16–18 d old) were transferred to the dark for 24 h to reduce the starch content of the chloroplasts. Tubes and solutions were sterilized and the intact chloroplasts were isolated on Percoll steps (21–60–80%) as aseptically as possible following the procedure described previously (14). After centrifugation for 12 min at 1200*g*, intact chloroplasts were collected from the 80% surface, Percoll was removed, and the chloroplasts were incubated at 25 ± 0.5°C in the dark or in the light (60 $\mu E\ m^{-2}\ s^{-1}$). After incubation, a second isolation of intact chloroplasts was carried out on Percoll step gradients (21 and 80%) by centrifugation at 900*g* for 6 min. Intact chloroplasts were collected from the 21 to 80% Percoll interface.

Measurement of Marker Enzymes, Chl, and Protein

Cyt *c* oxidase activity was determined by monitoring the absorbance at 547 nm (18). Acid phosphatase activity was determined with *p*-nitrophenyl-phosphate as described previously (16). Chloroplast suspension (2 or 5 μL) was mixed with 1 mL of 80% acetone and Chl was quantified according to Strain et al. (19). For the measurement of stromal protein content, chloroplasts were osmotically ruptured by mixing with 10 volumes of water on ice. After centrifugation, the concentration of protein in the supernatant was determined by the method of Bradford (2) using γ-globulin as the standard (Bio-Rad).

SDS-PAGE and Immunoblotting

Gel electrophoresis was carried out according to Laemmli (9) using 0.75-mm thick slab gels (12.5%). An aliquot of the

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[2] Abbreviations: PRK, phosphoribulokinase; GS, glutamine synthetase; NiR, nitrite reductase; LS, large subunit of Rubisco.

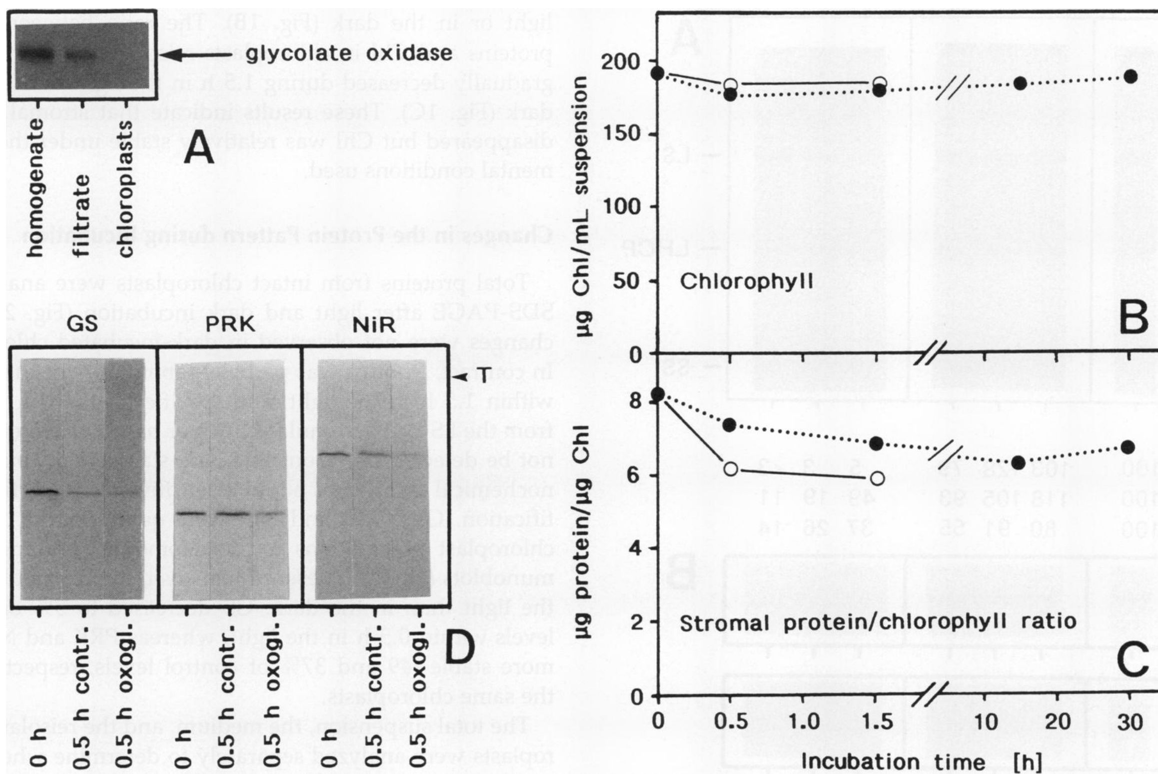

Figure 1. Control experiments with isolated chloroplasts. Glycolate oxidase was used as a marker for peroxisome contamination (A). The crude homogenate, the Miracloth filtrate, and the isolated chloroplasts were analyzed by immunoblotting with 1 μg of Chl/lane. The total Chl content in the suspension (B) and the stromal protein/Chl ratio in intact chloroplasts isolated after incubation (C) were measured in samples kept in the light (○) or in the dark (●). The effect of 1 mM 2-oxoglutarate (oxogl.) on the catabolism of selected stromal proteins was detected on immunoblots (D). After incubation for 0.5 h in the light, intact chloroplasts were collected from Percoll step gradients. A control without solutes (contr.) was included. Each lane was loaded with 0.3 μg of Chl (for PRK and GS) or 0.6 μg of Chl (for NiR). The top of the resolving gel is indicated (T).

chloroplast fraction was diluted with an equal volume of sample buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue) and boiled for 3 min. The samples were centrifuged to remove residual Percoll, and the supernatant was subjected to SDS-PAGE analysis. After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (0.45 μm; Bio-Rad) by the method of Towbin et al. (23). The membranes were blocked for 2 h with 1% (w/v) ovalbumin in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl). The blots were then incubated for 2 h with specific antibodies raised in rabbit (serum diluted 1:2000 for GS and glycolate oxidase; immunoglobulin G fraction diluted 1:1500 for NiR and 1:5000 for PRK). The membranes were treated for 2 h with bridging antibodies (anti-rabbit-immunoglobulin G developed in goat; Sigma) diluted 1:1000 and afterwards for 2 h with peroxidase-antiperoxidase complex (rabbit: Sigma) diluted 1:400. Peroxidase activity from the peroxidase-antiperoxidase complex was visualized with substrate solution (30 mg of 4-chloro-1-naphthol in 10 mL of methanol mixed with 50 mL of Tris-buffered saline and 15 μL of 30% H_2O_2). All steps were performed at room temperature. After blocking with ovalbumin, 0.05% (w/v) Tween-20 in Tris-buffered saline was used for washing (seven times for 5 min between all treatments mentioned above) as well as for the dilution of antibodies. The primary antibodies were produced by M. Höpfner, G. Ochs, and A. Wild, Mainz, Germany (against GS), by S.J. Crafts-Brandner, Lexington, KY (against PRK), by S. Ida, Kyoto, Japan (against NiR), and by Pel-Freez, Rogers, AR (against purified sugar beet glycolate oxidase purchased from Sigma). The bands on immunoblots were quantified by densitometric scanning (535 nm) with a CD 60 densitometer (Desaga, Heidelberg, Germany).

RESULTS

Purity and Integrity of the Isolated Chloroplasts

The purity of chloroplasts was judged by the activities or by immunostaining of marker enzymes. Cyt *c* oxidase was used as a mitochondrial marker and its activity indicated that mitochondrial contamination remained below 1% in the chloroplast fraction. Similarly, vacuolar contamination was negligible because less than 0.1% acid phosphatase activity was detectable after chloroplast isolation. Peroxisome contamination was analyzed by immunoblotting of glycolate oxidase. This polypeptide was not detectable immunologically in the isolated chloroplasts, although an intense band of glycolate

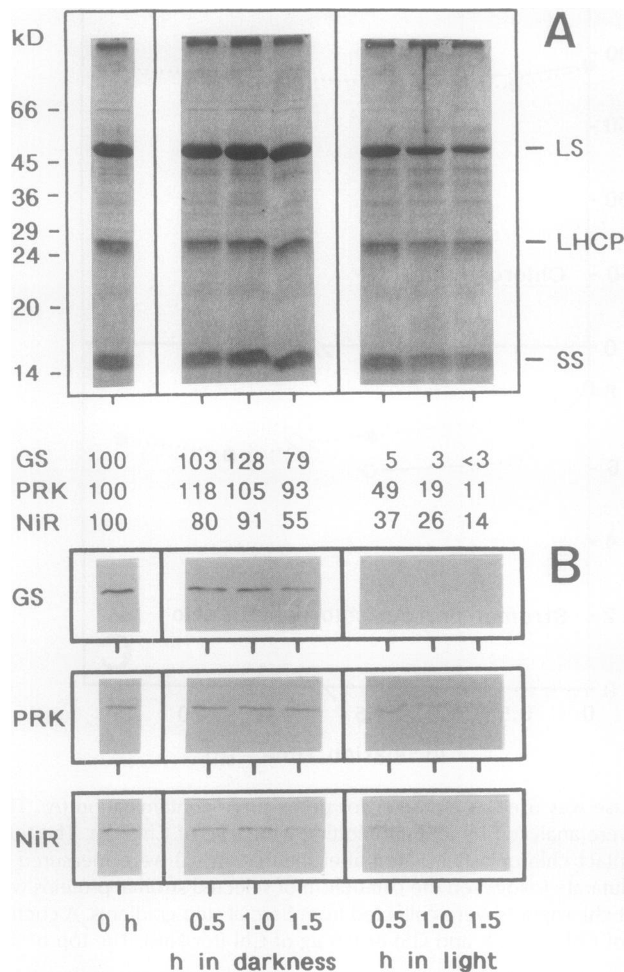

Figure 2. Changes in total chloroplast proteins during incubation in the light or dark. After incubation, intact chloroplasts were collected by centrifugation through Percoll steps. Total proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250 (A). Each lane was loaded with 0.6 μg of Chl to allow a direct comparison of the protein patterns before incubation (0 h) and after incubation in the dark or light. Molecular mass markers used were bovine α-lactalbumin (14 kD), soybean trypsin inhibitor (20 kD), trypsinogen from bovine pancreas (24 kD), bovine carbonic anhydrase (29 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), egg albumin (45 kD), and bovine albumin (66 kD). Selected stromal proteins were analyzed by immunoblotting with 0.3 (GS, PRK) or 0.6 (NiR) μg of Chl loaded per lane (B). The numbers above the lanes indicated the relative band staining (unincubated control = 100).

oxidase was present in the leaf homogenate (Fig. 1A). Contamination with nuclei or large membrane aggregates was not visible by phase contrast microscopy. Chloroplast integrity was tested by the latency of ferricyanide-reducing activity and by phase contrast microscopy (4). These criteria indicated that more than 90% of the freshly isolated and more than 70% of the reisolated chloroplasts were intact.

Changes in Chl and Stromal Proteins during Incubation

Only minor changes in total Chl content in the incubated chloroplast suspension were detected after incubation in the light or in the dark (Fig. 1B). The ratio between stromal proteins and Chl in chloroplasts reisolated after incubation gradually decreased during 1.5 h in the light or 30 h in the dark (Fig. 1C). These results indicate that stromal proteins disappeared but Chl was relatively stable under the experimental conditions used.

Changes in the Protein Pattern during Incubation

Total proteins from intact chloroplasts were analyzed by SDS-PAGE after light and dark incubation (Fig. 2). Major changes were not observed in dark-incubated chloroplasts. In contrast, Rubisco was partially (about 30% of the LS) lost within 1.5 h in the light, and specific breakdown products from the LS (14) accumulated. Minor bands of protein would not be detected on Coomassie blue-stained gels, but immunochemical techniques allow a sensitive and selective identification. GS, PRK, and NiR were quantified in the same chloroplast preparations by densitometric scanning of immunoblots (Fig. 2). These proteins were more rapidly lost in the light than in the dark. GS decreased to 5% of control levels within 0.5 h in the light, whereas PRK and NiR were more stable (49 and 37% of control levels, respectively) in the same chloroplasts.

The total suspension, the medium, and the reisolated chloroplasts were analyzed separately to determine whether the proteins were released into the external medium (Fig. 3).

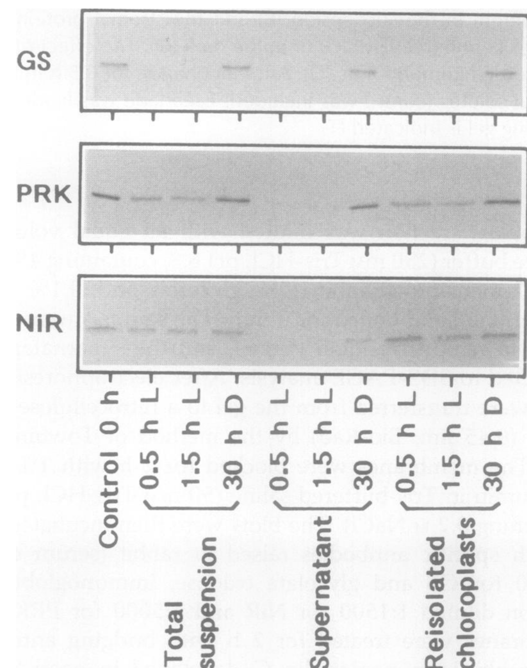

Figure 3. Loss of GS, PRK, and NiR in pea chloroplasts incubated in the light (L) or in the dark (D). The proteins were visualized by immunoblotting with specific antibodies. Each lane was loaded with 1 μg of Chl for the control (freshly isolated chloroplasts), the total suspension (including medium and chloroplasts), and the reisolated intact chloroplasts. Lanes with supernatant were loaded with the external medium equivalent to 1 μg of Chl in the total suspension prior to centrifugation.

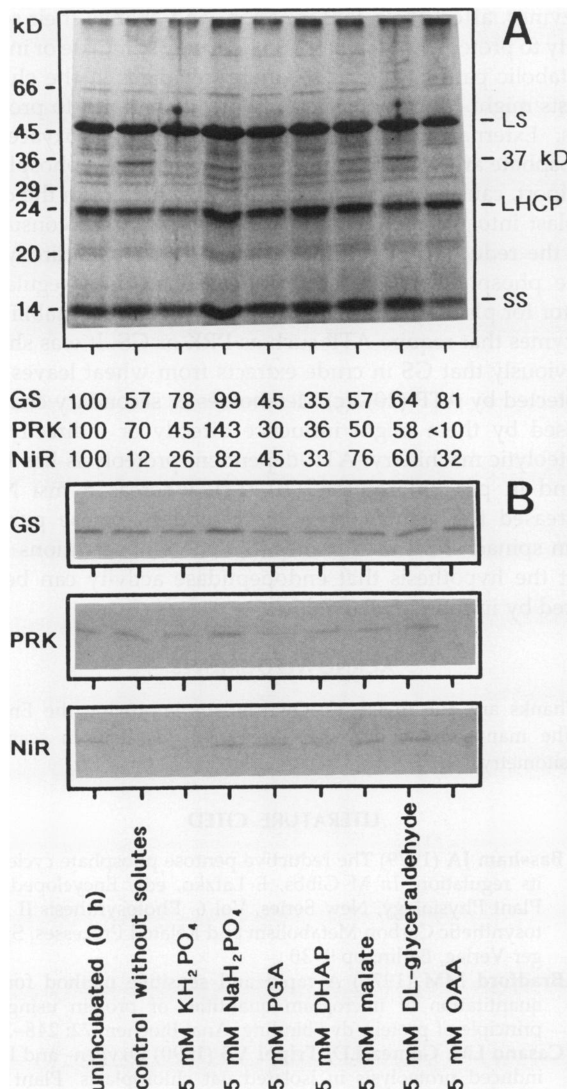

Figure 4. Effect of external solutes on plastid protein profiles in dark-incubated chloroplasts. Isolated chloroplasts were incubated with various solutes for 30 h in the dark. Intact chloroplasts were collected from Percoll steps after incubation. The protein pattern was analyzed by SDS-PAGE (A). Each lane was loaded with 0.6 μg of Chl. The molecular mass markers used are listed in the legend to Figure 2. Selected stromal proteins were analyzed by immunoblotting with 0.2 (PRK) or 0.3 (GS, NiR) μg of Chl loaded per lane (B). The numbers above the lanes indicate the relative band staining (unincubated control = 100). The initial chloroplast preparation (0 h) and a control incubated without solutes were included. The solutes indicated were added to the standard incubation medium.

Immunoreaction at the top of the gel and polypeptides deriving from the catabolism of GS, PRK, or NiR were not detected in the samples analyzed (Fig. 1D). These results indicate that the proteins were degraded in the light and not insolubilized or released into the external medium. The absence of immunoreactive breakdown products may be caused by a rapid further hydrolysis of the fragments produced by the initial cleavage. The presence of the enzymes in the supernatant after 30 h in the dark was caused by a high percentage of burst chloroplasts, but the reisolated intact chloroplasts still contained all three enzymes investigated (Fig. 3). The quantities of the proteins in the different fractions after 30 h in the dark suggest that the enzymes were quite stable in the external medium as well as within the chloroplasts in the absence of light.

Effect of External Solutes on Protein Catabolism in Chloroplasts

The composition of the external medium influenced the degradation of stromal proteins (Figs. 4 and 5). A 37-kD polypeptide deriving from the LS (14) accumulated under certain conditions after 30 h in the dark (Fig. 4). Other breakdown products from LS (14) became visible after incubation in the light (Fig. 2), but in this case external solutes caused no major differences in the polypeptide pattern on Coomassie blue-stained gels (data not shown). GS, PRK, and NiR were in general more rapidly degraded than Rubisco (Figs. 2 and 4). The effects of some solutes in the external medium on the catabolism of the three nuclear-encoded

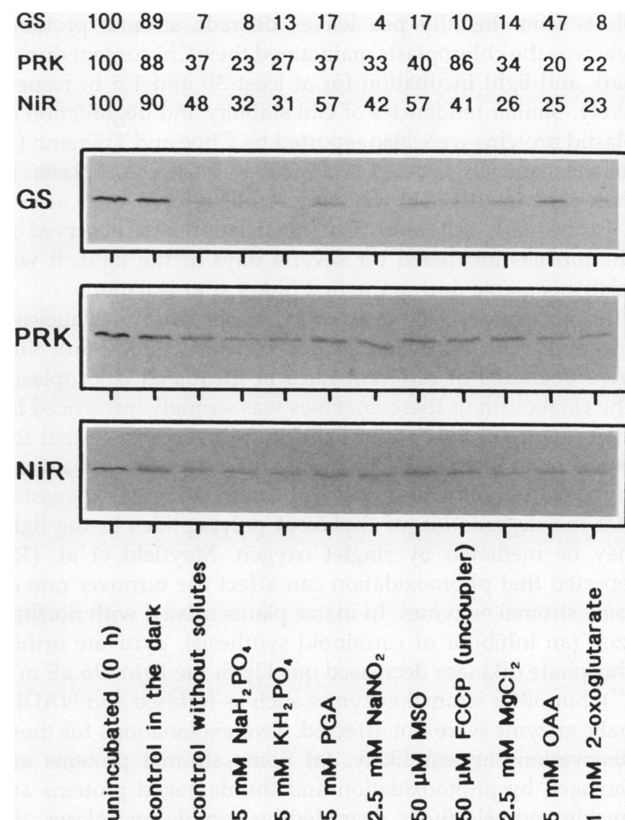

Figure 5. Effect of external solutes on the catabolism of GS, PRK, and NiR in isolated chloroplasts incubated with various solutes for 1.5 h in the light. Intact chloroplasts collected from Percoll steps after incubation were analyzed for GS, PRK, and NiR by immunoblotting. Each lane contained 0.3 (PRK, GS) or 0.6 (NiR) μg of Chl. The numbers above the lanes represent the relative band staining (unincubated control = 100). An unincubated control (0 h), a control incubated for 1.5 h in the dark without solutes, and a control incubated in the light without solutes were included.

enzymes depended on the presence or absence of light (Figs. 4 and 5). For example, in the light KH_2PO_4 and NaH_2PO_4 initiated a more rapid decrease of PRK and NiR (Fig. 5), whereas in the dark NaH_2PO_4 increased the stability of all three enzymes and no clear tendency was detected for KH_2PO_4. The effect of some solutes on the stability of the three enzymes was similar. For example, an accelerated degradation in the presence of 2-oxoglutarate in the light was detected after 0.5 h for GS, PRK, and NiR (Fig. 1D). This effect was still visible after 1.5 h for PRK and NiR, but in the case of GS the control was already too low (Fig. 5). Other solutes influenced the catabolism of the three nuclear-encoded stromal proteins differently. For example, oxaloacetic acid accelerated the catabolism of PRK in the dark (Fig. 4) and in the light (Fig. 5), but GS was stabilized under the same conditions. This interesting point was repeated in independent experiments. Our results indicate that certain metabolites may accelerate the degradation of one enzyme while delaying the degradation of a second enzyme.

DISCUSSION

Our experiments show that isolated and washed chloroplasts from healthy pea leaves degrade stromal proteins, whereas the chloroplasts maintained their Chl content during dark and light incubation for at least 30 and 1.5 h, respectively. Similar tendencies of Chl stability and degradation of plastid proteins were also reported by Choe and Thimann (4) for mechanically isolated and incubated oat chloroplasts. In their experiments, Chl was very stable at 26°C even after 7 d in the dark. Although Chl degradation was observed in chloroplasts incubated for several days in the light, it was relatively stable during the first hours after isolation.

In our experiments, it was further observed that nuclear-encoded stromal proteins, such as plastid GS, PRK, and NiR, were degraded at different rates in incubated chloroplasts. The catabolism of these enzymes was strongly influenced by the presence or absence of light. It has been known that the exposure of thylakoids to light causes the loss of several thylakoid polypeptides. Halloway and Dalling (6) suggested that the degradation of thylakoid polypeptides in the light may be mediated by singlet oxygen. Mayfield et al. (13) reported that photooxidation can affect the turnover rate of some stromal enzymes. In maize plants treated with norflurazon (an inhibitor of carotinoid synthesis), pyruvate orthophosphate dikinase decreased quickly in the light (96 $\mu E\ m^{-2}\ s^{-1}$), but other stromal enzymes such as Rubisco and NADP-malic enzyme were not affected. Two explanations for these observations appear likely. (a) Some stromal proteins are damaged by photooxidation and the damaged proteins are quickly and selectively degraded by peptide hydrolases. (b) An unknown endopeptidase is activated by a photochemical reaction and attacks the damaged or undamaged proteins. Recently, Casano et al. (3) found that an endopeptidase associated with thylakoid membranes was activated by hydrogen peroxide. The extent to which the two possibilities are relevant in vivo remains open to further study.

There are a number of ways in which external solutes can change the degradation rates for stromal proteins as observed in this paper. (a) Some metabolites may directly interact with enzymes, affect their conformation, and change their sensitivity to proteolytic attack. (b) Solutes may stimulate or inhibit metabolic pathways, and secondary changes in the chloroplasts might control the susceptibility of proteins to proteolysis. External phosphoglyceric acid or dihydroxyacetone phosphate are imported into the chloroplast by a phosphate-antiport, causing an efflux of orthophosphate from the chloroplast into the medium. ATP and NADPH are consumed for the reduction of phosphoglyceric acid to dihydroxyacetone phosphate in the stroma. Phosphate is a regulatory factor for photosynthesis, and ATP can affect the stability of enzymes that require ATP such as PRK or GS. It was shown previously that GS in crude extracts from wheat leaves was protected by ATP (20). (c) Metabolites or secondary changes caused by them might influence directly or indirectly the proteolytic machinery. ATP-dependent proteolysis has been found in pea chloroplasts (10, 12), whereas 5 mM NaCl decreased the activity of a 39-kD endopeptidase purified from spinach PSII membranes (8). These observations support the hypothesis that endopeptidase activity can be affected by intraorganellar solutes.

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