We have studied the turnover of an abundant chloroplast protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP\textsubscript{c} carboxylase/oxygenase), in plants (Spirodela oligorrhiza and Triticum aestivum L.) and algae (Chlamydomonas reinhardtii and C. moewusii) induced to senesce under oxidative conditions. RuBP\textsubscript{c} carboxylase/oxygenase activity and stability in vivo were found to be highly susceptible to oxidative stress, resulting in intermolecular cross-linking of large subunits by disulfide bonds within the holoenzyme, rapid and specific translocation of the soluble enzyme complex to the chloroplast membranes, and finally protein degradation. The redox state of Cys-247 in RuBP\textsubscript{c} carboxylase/oxygenase large subunit seems involved in the sensitivity of the holoenzyme to oxidative inactivation and cross-linking. However, this process did not drive membrane attachment or degradation of RuBP\textsubscript{c} carboxylase/oxygenase in vivo. Translocation of oxidized RuBP\textsubscript{c} carboxylase/oxygenase to chloroplast membranes may be a necessary step in its turnover, particularly during leaf senescence. Thus, processes that regulate the redox state of plant cells seem closely intertwined with cellular switches shifting the leaf from growth and maturation to senescence and death.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP\textsubscript{c} carboxylase/oxygenase)\textsuperscript{3} (EC 4.1.1.39) is a highly abundant bifunctional protein that catalyzes two competing reactions in the stroma of the chloroplasts, viz. photosynthetic CO\textsubscript{2} fixation and photorespiratory carbon oxidation. RuBP\textsubscript{c} carboxylase/oxygenase has been studied extensively from photosynthetic bacteria and algae to higher plants (1–3). The crystal structure of the bacterial protein has been solved recently (4, 5). RuBP\textsubscript{c} carboxylase/oxygenase from most prokaryotes and eukaryotes is a hexadecamer composed of eight chloroplast-encoded large (52–55 kDa) and eight nuclear-encoded small (12–18 kDa) subunits (1–3). Assembly of the oligomeric RuBP\textsubscript{c} carboxylase/oxygenase in plastids, or in a heterologous system such as Escherichia coli, is promoted by a class of proteins called chaperonins; viz. the large subunit-binding protein in plants and heat shock groEL/groES proteins in E. coli (6, 7).

Although much insight into transcriptional, translational, and post-translational regulation of synthesis, assembly, and activity of RuBP\textsubscript{c} carboxylase/oxygenase has been gained (1–8), our understanding of other important aspects of its function is not well defined. For instance, the protein accounts for about 40–50% (w/w) of soluble chloroplast protein that accumulates during leaf expansion, mainly because of high rates of its synthesis, with minimal, almost unmeasurable, degradation. But soon after leaf expansion ceases and senescence ensues RuBP\textsubscript{c} carboxylase/oxygenase is rapidly degraded concomitant with a marked decrease in CO\textsubscript{2} assimilation rates (9–13). The onset of degradation of RuBP\textsubscript{c} carboxylase/oxygenase and other proteins during leaf senescence has been speculated to provide nitrogen in the form of amino acids to young, developing leaves for growth (14). Thus, the senescing leaf has been used as a model for studying protein turnover (12). The recognition that protein turnover can occur within the chloroplast in opposition to the “lysosome” concept involving vacuolar proteases has shifted the emphasis from the vacuole to the photosynthetic organelle for studying RuBP\textsubscript{c} carboxylase/oxygenase degradation (15). However, little is known about the pathway or signals that trigger RuBP\textsubscript{c} carboxylase/oxygenase degradation. Studying RuBP\textsubscript{c} carboxylase/oxygenase degradation during whole plant senescence is problematic, in part because of the long duration of the process and difficulties in analyzing pulse-chase experiments in the face of contributions from changing developmental processes. Detached leaves or leaf discs have frequently been used in place of whole plants, but results obtained from using such material confounds analysis because of the additional effects of injury. Alternatively, senescence is enhanced by subjecting plants to environmental or chemical stresses that, in turn, lead to inactivation or degradation of RuBP\textsubscript{c} carboxylase/oxygenase (10, 16).

During chemical stress imposed by relatively high concentrations of cupric ions, higher plants undergo rapid physiological/biochemical changes comparable to those observed during normal senescence (17). We have used cupric ion-induced senescence in intact Spirodela oligorrhiza and wheat (Triticum aestivum L.) plants and purified wheat chloroplasts to analyze RuBP\textsubscript{c} carboxylase/oxygenase metabolism. We report here that oxidative conditions result in cross-linking of RuBP\textsubscript{c} carboxylase/oxygenase via disulfide bridges, translocation of the protein to chloroplast membranes, and rapid degradation of the protein. We also show that the redox state of Cys-247...
in the large subunit contributes to the sensitivity of Rbu-P₂ carboxylase/oxygenase to oxidative inactivation and cross-linking and that this process is not coupled to membrane translocation or degradation of Rbu-P₂ carboxylase/oxygenase in vivo.

**Experimental Procedures**

*Plant Material and Treatment with Cupric Ions—* Axenic cultures of *S. oleracea* (Kurtz) Hegelm. were grown phototrophically (15-20 μm m⁻² s⁻¹, 400-700 nm light, 28°C) for 10-15 days in half-strength Schramm’s mineral medium containing 1% sucrose.* Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* were grown in midlog phase in TAP medium containing 1% sucrose. Spike plants were removed from the vermiculite, roots carefully cleaned with water or 10 mM copper sulfate. These plants were washed two times in the homogenization medium containing 10 mM copper sulfate. Washing was continued with distilled water, and the plantlets were placed in a beaker containing water or 10 mM copper sulfate. These plantlets were incubated for 19 h. At 25°C under 15-20 μm m⁻² s⁻¹, 400-700 nm light. Isolated wheat chloroplasts were washed and pelleted as described below and the pellets resuspended in sorbitol/Hepes, pH 8, with or without 1 mM copper sulfate.

**Isolation of Wheat Chloroplasts—** Intact wheat chloroplasts were isolated as described (20) using a Percoll gradient of 10-90%. The chloroplasts were resuspended in 330 mM sorbitol and 50 mM Hepes/KOH, pH 8.0. Wheat chloroplast stroma and membranes were isolated as follows. After each treatment the chloroplasts were pelleted by letting the centrifuge accelerate to 3,500 g, followed immediately by deceleration. The intact chloroplast pellet was resuspended in the homogenization buffer and vortexed. The stroma fraction was separated from the membranes by centrifugation at 6,750 × g for 10 min. The membranes were washed as described below for total *Spirodela* membranes.

**In Vivo Labeling—** Prior to incubation with [³H]leucine (143.7 Ci/mmol; Du Pont-New England Nuclear) for radiolabeling proteins, *Spirodela* plants were transferred for 24 h to the medium without sucrose. Conditions for protein labeling and in vivo chase of radiolabeled proteins are described in the legend to Fig. 3.

**Isolation of Soluble and Membrane-associated Proteins—** *Spirodela* fronds and wheat leaves were homogenized in a medium containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 5 μM β-mercaptoethanol. Soluble and membrane fractions were isolated as described previously (21). Membrane fractions were washed twice in the homogenization medium containing 500 mM NaCl followed by resuspensions in 10 mM Tris-HCl, pH 8.0, and 10 mM Tris-HCl, pH 7.5. *C. reinhardtii* and *C. moewusii* cells were pelleted by centrifugation at 2,000 × g for 5 min, washed twice in ice-cold TAP medium, and then resuspended to obtain a chlorophyll concentration of 65 μg/ml. After treatment with 1 mM CuSO₄ in TAP medium, the cells were pelleted, resuspended in the homogenization medium described above, and broken in an ice-cold French press at 20,000 p.s.i. Soluble and membrane fractions were separated and washed as described above. Protein content in soluble fractions was determined by the dot blot assay (22) or Bio-Rad color reaction using bovine serum albumin as a standard. Membrane fractions were equalized on the basis of chlorophyll content determined by the standard method (23).

**Gel Electrophoresis and Immunoblotting—** Proteins were fractionated using non-denaturing polyacrylamide (7%) gels and denaturing SDS-polyacrylamide 10-20% gradient gels. Each sample was applied to the gels on either equal protein (soluble proteins) or equal chlorophyll (membrane proteins) or equal radioactivity bases as indicated in the legend to each figure. A part of each gel was stained with Coomasie Blue or fluorographed and the remaining parts electrotransferred onto nitrocellulose for immunodetection (24) with antisera against specific proteins as described in the appropriate figure legend.

Each gel and immunoblot were repeated a minimum of four times. Reproducible results were obtained each time, and typical results are presented here.

**Results**

*Inactive Rbu-P₂ Carboxylase/Oxygenase Associates with Membranes—* *Spirodela*, wheat plants, and wheat chloroplasts were incubated with or without copper sulfate for different times, following which soluble and membrane-associated proteins were isolated and fractionated by SDS-PAGE. The results, shown in Fig. 1, indicate a steady decline in the levels of soluble proteins fractionating at about 55 kDa and 14 kDa in plants treated with cupric ions concomitant with an increased accumulation of similar size proteins in the membrane fraction (Fig. 1A). Since large and small subunits of Rbu-P₂ carboxylase/oxygenase, respectively, fractionate at 55 and 14 kDa during SDS-PAGE, immunodetection using anti-Rbu-P₂ carboxylase/oxygenase antibodies was carried out. It is clear from Fig. 1B that the 55- and 14-kDa proteins in the soluble pool which are markedly affected by cupric ion toxicity indeed represent large and small subunits of Rbu-P₂ carboxylase/oxygenase, respectively. These results are consistent with previous observations of increased in vitro proteolysis of large subunit of Rbu-P₂ carboxylase/oxygenase under oxidative treatments (25). The presence of lower molecular weight polypeptides immunoreactive with antibodies against Rbu-P₂ carboxylase/oxygenase, particularly in isolated wheat chloroplasts, is further evidence of degradation of large subunit of Rbu-P₂ carboxylase/oxygenase under these conditions (Fig. 1C, compare lanes 6-8 with lane 5). Inability to detect discrete breakdown products of Rbu-P₂ carboxylase/oxygenase in *Spirodela* or intact wheat plants may be caused by faster rates of degradation and/or loss of immunological epitopes in the breakdown products. However, association of these proteins with membranes was somewhat surprising. Therefore, to rule out nonspecific adhesion of Rbu-P₂ carboxylase/oxygenase to membranes, the membrane fractions were thoroughly washed with 0.3 and 1.5 M NaCl as well as with low ionic strength buffers at pH values of 7.5 and 8.0. None of these treatments was effective in dissociating the immunoreactive Rbu-P₂ carboxylase/oxygenase subunits from the membranes.

The disappearance of soluble *Spirodela* and wheat Rbu-P₂ carboxylase/oxygenase subunits closely correlated with a corresponding decrease in the Rbu-P₂ carboxylase/oxygenase carboxylation activity which was, however, not recovered with the membranes (data not shown), suggesting that membrane-associated Rbu-P₂ carboxylase/oxygenase did not represent an active enzyme.

**Steady-state Level of Soluble Rbu-P₂ Carboxylase/Oxygenase Decreases during Cupric Ion Toxicity—** The translation of the two subunits to the membranes increased upon incubation of *Spirodela* (Fig. 1A, lanes 6-10), wheat plants (Fig. 1B, lanes 3 and 4), and wheat chloroplasts (Fig. 1C, lanes 5-8) for prolonged periods with cupric ions (Fig. 1B). The quantified data for the membrane translocation kinetics of large subunit of Rbu-P₂ carboxylase/oxygenase in *Spirodela* and wheat chloroplasts are presented in Fig. 2. A relatively small, but measurable, amount of Rbu-P₂ carboxylase/oxygenase was found consistently associated with the membranes from control plants as well (Fig. 1A, lane 6; Fig. 1B, lane 3).

To determine if the changes observed in the steady-state levels of proteins were caused by differential degradation rates, pulse-chase experiments were carried out. Plants were pulse labeled for 3 h with [³H]leucine, and then radioactivity in proteins was chased for different time periods with or without 1 mM cupric sulfate in the mineral medium containing nonradioactive leucine (1 mM). Soluble and membrane proteins were isolated and fractionated by SDS-PAGE. Fluorographs of these gels are presented in Fig. 3.

Pulse-chase experiments showed that soluble proteins turn
Fig. 1. Association of Rbu-P₂ carboxylase/oxygenase with membranes during cupric ion-induced oxidative stress in intact Spirodela plants (A), wheat plantlets (B), and isolated wheat chloroplasts (C). Spirodela plants were incubated in the absence (4 h) (lanes 1 and 6) and presence (lanes 2-5 and 7-10) of 1 mM CuSO₄ for 0.25 h (lanes 2 and 7), 1 h (lanes 3 and 8), 2 h (lanes 4 and 9), and 4 h (lanes 5 and 10). Wheat plantlets were given 10 mM CuCl₂ (B, lanes 2 and 4) or H₂O (lanes 1 and 3) for 19 h. Isolated wheat chloroplasts were incubated in the absence (1 h) (C, lanes 1 and 5) and presence of 1 mM CuSO₄ for 0.25 h (C, lanes 2 and 6), 0.5 h (C, lanes 3 and 7), and 1 h (C, lanes 4 and 8). After incubation, soluble (S) and membrane (M) proteins were isolated, fractionated by SDS-PAGE, and either stained with Coomassie Blue (Stain) or electrotransferred onto nitrocellulose paper and immunoreacted with antibodies against Rbu-P₂ carboxylase/oxygenase (Blot). Equal amounts of soluble (6 µg/lane) and membrane (1 µg of chlorophyll equivalent/lane) proteins were applied on the gels. The positions of molecular weight standards and of the large subunit (LS) and the small subunit (SS) of Rbu-P₂ carboxylase/oxygenase are indicated.

Fig. 2. Quantification of large subunits of Rbu-P₂ carboxylase/oxygenase in soluble and membrane fractions of Spirodela and wheat chloroplasts as a function of incubation time with 1 mM CuSO₄. Coomassie-stained gels (represented in Fig. 1, A and C) were scanned using an LKB laser densitometer (24). The area of the soluble and membrane-associated large subunit (LS) of Rbu-P₂ carboxylase/oxygenase was normalized and the maximum given an arbitrary value of 100. The values obtained in three separate scans were plotted as large subunit area against time of incubation with cupric ions. Curve fitting was done using Cricket graph software in Macintosh computer. Regression coefficient (r) values were 0.91 and 0.82 for Spirodela membrane and soluble fractions, respectively, and 0.91 and 0.97 for wheat chloroplast membrane and soluble fractions, respectively. Closed circles, soluble large subunit; open triangles, membrane-associated large subunit.

Fig. 3. Cupric ion-induced breakdown of Rbu-P₂ carboxylase/oxygenase and its transient association with membranes. Spirodela plants were radiolabeled for 3 h with 25 µCi/ml [³H]leucine (Amersham Corp.) in sucrose-free mineral medium and then washed and incubated in the light with fresh medium containing nonradioactive leucine (1 mM) with or without 1 mM CuSO₄. Samples were removed at 0, 2, 4, 8, and 18 h of chase and homogenized. Soluble and membrane-associated proteins were fractionated on SDS-PAGE and fluorographed (22). The positions of large subunit (LS) and small subunit (SS) of Rbu-P₂ carboxylase/oxygenase, the 32-kDa protein of the photosystem II (32), and the light-harvesting chlorophyll a/b apoprotein (LHCP) are indicated. Samples applied on the gels contained 20,000 cpm (soluble fraction) or 80,000 cpm (membrane fraction) of hot trichloroacetic acid-precipitable radioactivity.

over more rapidly in Spirodela incubated with cupric ions (for > 4 h) than in the control plants incubated without CuSO₄; in particular the turnover of Rbu-P₂ carboxylase/oxygenase was more striking (Fig. 3, Soluble). However, some amount of radiolabeled Rbu-P₂ carboxylase/oxygenase was found associated with membranes only in samples incubated for 8 and 18 h (Fig. 3, Membrane). Clearly, the membrane-associated radiolabeled Rbu-P₂ carboxylase/oxygenase also turned over appreciably in the 18-h sample. These results are different from those of the steady-state distribution of Rbu-P₂ carboxylase/oxygenase during cupric ion treatment (Fig. 1A), indicating that membrane association of Rbu-P₂ carboxylase/oxygenase is transient and that membrane-associated Rbu-P₂ carboxylase/oxygenase undergoes turnover. Also evident from data in Fig. 3 is that, among other changes, the degradation of light harvesting chlorophyll a/b apoprotein, and the D1 (marked 32) protein of photosystem II is also enhanced.

Steady-state membrane protein levels have been reported to change at different rates during senescence (26, 27). Similarly, cupric ions cause inactivation of chloroplast photosystems of some (28, 29) but not all plants (30). However, in contrast to the effect on Rbu-P₂ carboxylase/oxygenase demonstrated above, the steady-state levels of several chloroplast membrane proteins did not change as markedly within the time frame of cupric ion treatment of intact plants, results that are consistent with previous observations using intact spinach plants (30). This is particularly evident from immunoblots for the extrinsic 33-kDa oxygen-evolving complex
protein, light-harvesting chlorophyll a/b apoprotein, subunit 1 of the photosystem I, 32-kDa D1 protein of the photosystem II, and plastocyanin (24; Fig. 4). The β-subunit of ATPase, on the other hand, declined in abundance as the treatment of *Spirodela* with cupric ions increased to 4 h. Similar instability of CF1-ATPase was shown to occur during senescence of wheat leaves (31) but not in oat leaves (26).

Overall, these results indicate that the majority of photosynthetic membrane proteins remain more or less unaffected during the early period (several hours) of cupric ion-induced senescence. Thus, one of the earliest and most profound consequences of this phenomenon is the instability of Rbu-P$_2$ carboxylase/oxygenase and its translocation from the soluble fraction to the membrane fraction of the chloroplast.

**Dimerization of Rbu-P$_2$ Carboxylase/Oxygenase Involving Sulfhydryl Group(s) Occurs in Concert with Its Membrane Translocation**—What triggers the disappearance and membrane translocation of Rbu-P$_2$ carboxylase/oxygenase in cupric ion-treated plants? Cupric ions, as transition elements, are strong oxidants (32) and can change oxidation-reduction potential in a biological environment. This in turn might adversely affect macromolecules such as proteins, the degree of damage being dependent upon the *micro milieu* and the presence of, yet to be defined, sensitive amino acid residues (33). Since large subunits of Rbu-P$_2$ carboxylase/oxygenase have been shown to cross-link in vitro (34, 35), we sought to check the possibility that oxidative conditions caused by cupric ions might result in the *in vivo* cross-linking of Rbu-P$_2$ carboxylase/oxygenase molecules involving cysteine residues.

*Spirodela*, wheat plants, and isolated wheat chloroplasts were incubated with or without CuSO$_4$, and soluble proteins were isolated. Samples were then boiled with the sample application buffer with or without β-mercaptoethanol and fractionated by SDS-PAGE. Results are presented in Fig. 5. The elimination of β-mercaptoethanol from a parallel set of samples was expected to maintain sulfhydryl groups in an oxidized configuration and thus enable cross-linked proteins to electrophorese more slowly under nonreducing but denaturing conditions. Indeed, we found a protein band of ~110–120 kDa in cupric ion-treated *Spirodela* and wheat chloroplast samples electrophoresed under nonreducing conditions, the appearance of which occurred concomitant with the disappearance of the large subunit at ~55 kDa (Fig. 5). In the treated intact wheat samples, both the protein bands appeared to be absent. This was attributed to longer time period used in this experiment which resulted in the degradation of both the protein forms, since in short term experiments with intact wheat plantlets, we did observe a trend similar to that in *Spirodela* and wheat chloroplasts. The ~110–120 kDa protein, representing a large subunit dimer, was either absent or present in very low levels in the nontreated controls. When identical cupric ion-treated samples were fractionated under reducing conditions, the doublet disappeared (cf. Fig. 1). The concentration of the Rbu-P$_2$ carboxylase/oxygenase small subunit appeared not to change under these conditions (data not shown). These results suggest that large subunits in the holoenzyme are cross-linked *in vivo* under oxidative conditions.

If large subunits of the Rbu-P$_2$ carboxylase/oxygenase holoenzyme were oxidized and then cross-linked via disulfide bonds, we surmised that the oxidized holoenzyme might be separable from the unoxidized form under nondenaturing conditions. Thus, soluble proteins from control and cupric ion-treated *Spirodela* plants were fractionated on 7% polyacrylamide gels under nondenaturing conditions and either stained with Coomassie Blue (Fig. 6A, lanes 1–5) or immunoblotted (Fig. 6A, lanes 6–10). A perceptible shift in the mobility of Rbu-P$_2$ carboxylase/oxygenase as seen in the Western blot analysis using antibodies against Rbu-P$_2$ carboxylase/oxygenase appears to occur during senescence of wheat leaves (31) but not in oat leaves (26).

FIG. 4. Effect of cupric ion toxicity on the steady-state level of the indicated chloroplast proteins. *Spirodela* plants were incubated with 1 mM CuSO$_4$, for the indicated times (lanes 3–7). Chloroplast proteins were isolated, fractionated by SDS-PAGE, and immunoblotted. The blots were immunoreacted with antibodies against subunit 1 of photosystem I (PSI), β-ATPase, 33-kDa extrinsic photosystem II protein (33-KDa), light-harvesting chlorophyll a/b apoprotein (LHCP), and plastocyanin. Samples from control plants incubated without cupric ions are shown in lanes 1 and 2.

FIG. 5. Dimerization of Rbu-P$_2$ carboxylase/oxygenase large subunit in *Spirodela* plants, wheat plantlets, and isolated wheat chloroplasts upon oxidative stress. Plants or isolated chloroplasts as indicated were treated with CuSO$_4$, for 19 h (lane 7) in the case of wheat; or 0.25 h (lane 9) and 0.5 h (lane 10) in the case of isolated chloroplasts; or 0.25 h (lane 2), 1 h (lane 3), 2 h (lane 4), and 4 h (lane 5) in the case of *Spirodela* as described in the legend to Fig. 1. Soluble proteins (5 μg/lane) were prepared in sample buffer lacking β-mercaptoethanol, electrophoresed under nonreducing conditions, and immunoblotted. The immunoblots were reacted with anti-Rbu-P$_2$ carboxylase/oxygenase antibody. Lanes 1, 6, and 8 represent samples from control plants incubated without cupric ions. The upper and lower arrows on the right indicate the positions of large subunit dimer and large subunit monomer, respectively.
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The generality of disulfide cross-linking in Rbu-P\(_2\) carboxylase/oxygenase implies that Cys-247 and its micro milieu may have special characteristics that make the protein highly sensitive to oxidative conditions. In fact, Cys-247 is conserved in almost all the Rbu-P\(_2\) carboxylase/oxygenase large subunits sequenced thus far (37). An interesting exception is the \(C.\ moewusii\) protein, in which it is replaced by serine (38). It was, therefore, of interest to investigate if, in this alga, Rbu-P\(_2\) carboxylase/oxygenase is refractory to oxidative damage and if disulfide bridge formation is linked to membrane translocation of Rbu-P\(_2\) carboxylase/oxygenase.

Phototrophic cultures of \(C.\ reinhardtii\) and \(C.\ moewusii\) were incubated with or without 1 mM CuSO\(_4\) for 0.5-4 h, and cells were then harvested and lysed. Soluble and membrane proteins were isolated, fractionated on SDS-polyacrylamide gels under reducing and nonreducing conditions, and immunooblotted using a mixture of antibodies against Rbu-P\(_2\) carboxylase/oxygenase large and small subunits.

Under reducing conditions, cupric ion treatment of both algal cultures resulted in a time-dependent loss of the Rbu-P\(_2\) carboxylase/oxygenase subunits from the soluble pool and their translocation to the membranes (Fig. 7A). These data are consistent with other results presented above for \(S.\ moewusii\) and \(C.\ reinhardtii\). Moreover, when the same set of soluble samples in Fig. 7A was electrophoresed under nonreducing conditions (i.e., in the absence of DTT) and reacted with the anti-Rbu-P\(_2\) carboxylase/oxygenase antibodies, it became evident that Rbu-P\(_2\) carboxylase/oxygenase from \(C.\ reinhardtii\) was reversibly cross-linked via disulfide bonds (Fig. 7B, lanes 17-20) whereas Rbu-P\(_2\) carboxylase/oxygenase from \(C.\ moewusii\) was not and appeared impervious to oxidation stress in this regard (Fig. 7B, lanes 7-10). Nonetheless, even in the absence of cross-linking, \(C.\ moewusii\) Rbu-P\(_2\) carboxylase/oxygenase was found to translocate to membranes under these conditions (Fig. 7A). These results implicate the S-S cross-linking of Rbu-P\(_2\) carboxylase/oxygenase under oxidative damage to Cys-247 in the large subunit and further indicate that cross-linking at

\[\text{Rbu-P\(_2\) carboxylase/oxygenase}\]

...
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Cys-247 is not required for Rbu-P₂ carboxylase/oxygenase translocation to membranes. Thus, the two processes seem to occur independent of each other in vivo.

**DISCUSSION**

We have demonstrated that Rbu-P₂ carboxylase/oxygenase, an abundant chloroplast protein, is highly sensitive to oxidative stress. As a result, Rbu-P₂ carboxylase/oxygenase undergoes in vivo -S-S- cross-linking (most probably at Cys-247 of large subunit in the assembled holoenzyme), inhibition of enzyme activity, membrane translocation, and, finally, degradation. Further, our data show that membrane translocation of Rbu-P₂ carboxylase/oxygenase occurs independent of disulfide cross-linking. Thus, oxidative stress affects Rbu-P₂ carboxylase/oxygenase stability in more than one way.

The results described here may have relevance to the precipitous enhancement in the inactivation and degradation of Rbu-P₂ carboxylase/oxygenase protein during plant senescence. Since the redox state of Cys-247 seems to determine the sensitivity of Rbu-P₂ carboxylase/oxygenase to inactivation and cross-linking (Fig. 7; 25), a highly reduced environment must be maintained by the plastid to ensure the stability of Rbu-P₂ carboxylase/oxygenase during normal growth. When these protective mechanisms in the cell break down, for instance, during senescence and stress, a change in redox to a more oxidized state might result in the instability of Rbu-P₂ carboxylase/oxygenase. Indeed, indirect evidence has been presented to show that during senescence more oxidative conditions exist in the chloroplast (39). Furthermore, recently it has been reported that removing fruit from soybean plants causes formation of insoluble Rbu-P₂ carboxylase/oxygenase in leaf extracts (40). Thus, the oxidation-reduction state of the chloroplast stroma appears closely associated with shifts in the leaf from a normal growth/maturity stage to senescence and death.

Translocation of oxidized Rbu-P₂ carboxylase/oxygenase to the chloroplast membranes may be a mechanism for the regulation of its turnover, particularly during senescence. In its oxidized and membrane-associated conformation Rbu-P₂ carboxylase/oxygenase may be more prone to proteolysis. The nature and type of the protease that specifically recognizes Rbu-P₂ carboxylase/oxygenase in vivo during senescence and degrades it have remained puzzling questions about Rbu-P₂ carboxylase/oxygenase biology. Many studies have demonstrated involvement of proteases in the degradation of Rbu-P₂ carboxylase/oxygenase in vitro. However, none of these in vitro studies shows the specificity expected of an in vivo proteolytic system for Rbu-P₂ carboxylase/oxygenase (41–43). It is possible that it is the oxidized form of Rbu-P₂ carboxylase/oxygenase that is the actual/natural substrate for its specific protease, and the inability to find a Rbu-P₂ carboxylase/oxygenase-specific protease may be because the substrate tested in all such studies was the reduced protein. In this context, it is of interest that in vitro studies using general proteases, viz. trypsin, chymotrypsin, proteinase K, and papain, have shown enhanced degradation of the oxidized form compared with reduced Rbu-P₂ carboxylase/oxygenase as the substrate (25).

Difficulties encountered in the isolation of a Rbu-P₂ carboxylase/oxygenase-specific protease may also be linked to yet another possibility raised by the data reported here. Since oxidative conditions result in membrane translocation of Rbu-P₂ carboxylase/oxygenase prior to its degradation in vivo, it may be that Rbu-P₂ carboxylase/oxygenase degradation is in fact catalyzed by a membrane-associated protease rather than a stroma one. Alternatively, membrane association of oxidized Rbu-P₂ carboxylase/oxygenase may act as a scaffold providing the right conformation for a stroma or membrane protease to act. Implicit in such a possibility is the involvement of a membrane binding step for oxidized (damaged/modified) Rbu-P₂ carboxylase/oxygenase during its degradation. If this is the case, then oxidized Rbu-P₂ carboxylase/oxygenase will have higher affinity for the membrane than will the native, undamaged protein. Indeed, in our preliminary reconstitution experiments using salt-washed chloroplast membranes and gel-purified radiolabeled Rbu-P₂ carboxylase/oxygenase, we have found a higher affinity of oxidized, rather than reduced, Rbu-P₂ carboxylase/oxygenase protein for chloroplast membranes.

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**REFERENCES**

1. Miziolek, H. M., and Lorimer, G. H. (1983) Annu. Rev. Biochem. 52, 507–535

2. Paech, C. (1985) in Modern Methods of Plant Analysis, New Series, Vol. 1: Cell Components (Linskasen, H. F., and Jackson, J. F., eds) pp. 199–230, Springer-Verlag, New York

3. Ellis, R. J. (1985) in Molecular Biology of the Photosynthetic Apparatus, pp. 339–347, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

4. Chapman, M. S., Suh, S. W., Cascio, D., Smith, W. W., and Eisenberg, D. (1987) Nature 329, 354–356

5. Anderson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C.-I., and Lorimer, G. (1989) Nature 337, 229–234

6. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilley, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature 333, 390–394

7. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44–47

8. Avni, A., Edelman, M., Rachaiovich, I., Aviv, D., and Flhr, R. (1989) EMBO J. 8, 1915–1918

9. Newman, S. M., and Cattolico, R. A. (1990) Photosynth. Res. 26, 69–85

10. Peterson, L. W., and Huffaker, R. C. (1975) Plant Physiol. 55, 1009–1015

11. Wittenbach, V. A. (1978) Plant Physiol. 62, 604–606

12. Thimmann, K. V. (ed) (1980) Senescence in Plants, CRC Press, Boca Raton, FL

13. Maes, T., Makino, A., and Ohira, K. (1987) in Plant Senescence: Its Biochemistry and Physiology (Thomson, W. W., Nothnagel, E. A., and Huffaker, R., eds) pp. 123–131, The American Society of Plant Physiologists, Rockville, MD

14. Peoples, M. B., Beilharz, V. C., Waters, S. P., Simpson, R. J., and Dalling, M. J. (1980) Planta (Berl.) 149, 241–251

15. Dalling, M. J., and Nettleton, A. M. (1986) in Plant Proteolytic Enzymes (Dalling, M. J., ed) Vol. 2, pp. 125–153, CRC Press, Boca Raton, FL

16. Ferreira, R. B., and Davies, D. D. (1989) Planta (Berl.) 179, 448–455

17. Matteo, A. K., Baker, J. E., and Moline, H. E. (1985) J. Plant Physiol. 125, 193–202

18. Posner, H. B. (1967) in Methods in Developmental Biology (Witt, F. A., and Wassela, N. K., eds) pp. 301–317, Crowell, New York

19. Gorman, D. S., and Levine, R. P. (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 1665–1669

20. Bartlett, S. G., Grossman, A. R., and Chun, N.-H. (1982) in Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R. B., and Chun, N.-H., eds) pp. 955–1014, Elsevier Science Publishing Co., Amsterdam

21. Reisfeld, A., Matteo, A. K., and Edelman, M. (1982) Eur. J. Biochem. 124, 125–129
22. Marder, J. B., Mattoo, A. K., and Edelman, M. (1986) *Methods Enzymol.* **118**, 384–396
23. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
24. Callahan, F. E., Wergin, W. P., Nelson, N., Edelman, M., and Mattoo, A. K. (1989) *Plant Physiol.* **91**, 629–635
25. Penarrubia, L., and Moreno, J. (1990) *Arch. Biochem. Biophys.* **281**, 319–323
26. Ben-David, H., Nelson, N., and Gepstein, S. (1983) *Plant Physiol.* **73**, 507–510
27. Roberts, D. R., Thompson, J. E., Dumbroff, E. B., Gepstein, S., and Mattoo, A. K. (1987) *Plant Mol. Biol.* **9**, 343–353
28. Shio, Y., Tamai, H., and Sasa, T. (1978) *Plant Cell Physiol.* **19**, 203–209
29. Hsu, B-D., and Lee, J-Y. (1988) *Plant Physiol.* **87**, 116–119
30. Baszynski, T., Krol, M., Krupa, Z., Roszkowska, M., Wojcieska, U., and Wolinska, D. (1982) *Z. Pflanzenphysiol.* **108**, 385–388
31. Camp, P. J., Huber, S. C., and Moreland, D. E. (1984) *J. Exp. Bot.* **35**, 659–668
32. Loneragan, J. F., Robson, A. D., and Graham, R. D. (eds) (1981) *Copper in Soils and Plants*, Academic Press, New York
33. Stadtman, E. R., and Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005–2008
34. Rintamaki, E. (1989) *J. Exp. Bot.* **40**, 1305–1313
35. Newman, J., and Gutteridge, S. (1990) *J. Biol. Chem.* **265**, 15154–15159
36. Rathy, B., Lorimer, G., and Gutteridge, S. (1991) *Eur. J. Biochem.* **200**, 353–358
37. Hudson, G. S., Mahon, J. D., Anderson, P. A., Gibbs, M. J., Badger, M. R., Andrews, T. J., and Whitfield, P. R. (1990) *J. Biol. Chem.* **265**, 808–814
38. Yang, R. C. A., Dove, M., Seligy, V. L., Lemieux, C., Turmel, M., and Narang, S. A. (1986) *Gene (Amst.)* **50**, 259–270
39. McRae, D. G., and Thompson, J. E. (1983) *Planta (Berl.)* **158**, 185–198
40. Crafts-Brandner, S. J., Salvucci, M. E., and Egli, D. B. (1991) *Planta (Berl.)* **183**, 300–306
41. Storey, R. D., and Beevers, L. (1977) *Planta (Berl.)* **137**, 37–44
42. Feller, U. (1979) *Z. Pflanzenphysiol.* **95**, 413–422
43. Ragster, L. E., and Chrispeels, M. J. (1979) *Plant Physiol.* **64**, 857–862