A Proposed Mechanism for the Inhibitory Effects of Oxidative Stress on Rubisco Assembly and Its Subunit Expression[1]

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In *Chlamydomonas reinhardtii*, a light-induced oxidative stress shifts the glutathione pool toward its oxidized form, resulting in a translational arrest of the large subunit (LSU) of Rubisco. We show here that the translational arrest of LSU is tightly coordinated with cessation of Rubisco assembly, and both processes take place after a threshold level of reactive oxygen species is reached. As a result, the small subunit is also eliminated by rapid degradation. We previously showed that the amino terminus of the LSU could bind RNA in a sequence-independent manner, as it shares a structural similarity with the RNA recognition motif. This domain becomes exposed only under oxidizing conditions, thus restricting the RNA-binding activity. Here we show that in vitro, thiol groups of both subunits become oxidized in the presence of oxidized glutathione. The structural changes are mediated by oxidized glutathione, whereas only very high concentrations of H_2O_2 confer similar results in vitro. Changes in the redox state of the LSU thiol groups are also observed in vivo, in response to a physiological light shock caused by transfer of cells from low light to high light. We propose that during a photooxidative stress, oxidation of thiol groups occurs already in nascent LSU chains, perhaps hindering their association with chaperones. As a result, their RNA recognition motif domain becomes exposed and will bind any RNA in its vicinity, including its own transcript. Due to this binding the ribosome stalls, preventing the assembly of additional ribosomes on the transcript. Polysome analysis using Suc gradients indeed shows that the *rbcL* RNA is associated with the polysomal fraction at all times but shifts toward fractions that contain smaller polysomes and monosomes during oxidative stress. Thus, translational arrest of the LSU most likely occurs at a postinitiation stage.

Rubisco is responsible for CO_2 fixation during photosynthesis. In vascular plants and green algae, it exists as a holoenzyme composed of eight large subunits (LSUs; 55 kD) encoded by the chloroplast *rbcL* gene and eight small subunits (SSUs; 15 kD) produced by a nuclear family of *rbcS* genes (Spreitzer, 1993). SSU precursors are processed during entry into the chloroplast and are then assembled with the LSUs to yield the holoenzyme. Assembly of the oligomeric protein (approximately 500 kD) is mediated by cpn60 and cpn10 (Gatenby and Ellis, 1990), and a complex of the LSU and cpn60 serves as an intermediate of the assembly process. The chloroplast cpn60 is a homolog of bacterial groEL, and prokaryotic subunits of Rubisco expressed in *Escherichia coli* can be successfully assembled into a holoenzyme (Goloubinoff et al., 1989). A role for additional chaperone molecules, in accumulation of mature Rubisco complexes, was recently described (Brutnell et al., 1999).

Many protein complexes in the chloroplast are composed of multiple polypeptides, which are expressed in a tightly coordinated manner, as removal of one subunit can have an effect on expression of the other subunits in the complex. The coordinated expression of different subunits of the same complex can be achieved by controlled synthesis, as well as by proteolytic degradation of unassembled subunits. An assembly-dependent regulation was defined as a control by epistasy by Wollman and colleagues (Choquet et al., 1998; Wollman et al., 1999). In the case of cytochrome *f*, an autoregulatory mechanism was shown for the chloroplast-encoded *petA* gene, by which the C-terminal domain of the unassembled polypeptide had a negative feedback effect on translation of the *petA* mRNA (Choquet et al., 2003). A later study showed that components of the PSI system in *Chlamydomonas reinhardtii* are regulated at the level of translation initiation (Wostrikoff et al., 2004). Expression of Rubisco subunits is also tightly coordinated, and elimination of either subunit impinges on expression of its counterpart in the complex (Schmidt and Mishkind, 1983; Khrebtukova and Spreitzer, 1996; Rodermel et al., 1996). However, the fine details of the regulatory processes involved were not fully resolved.

Exposure of plant cells to excess illumination generates an oxidative stress due to an imbalance between the antennae size and the inability to dissipate the adsorbed energy (Shapira et al., 1997). As a result of transferring *C. reinhardtii* cells from low light (LL) to

[1] This work was supported by the Israel Science Foundation (grant no. 515/02).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.056341.

high light (HL), the level of oxygen radicals transiently increases, and the glutathione pool shifts toward its oxidized form (Irihimovitch and Shapira, 2000). Parallel to these effects, we previously showed that translation of the Rubisco LSU stops and resumes only when the cells recover from this stress. This pattern of regulation is unique to Rubisco LSU and was not observed with any other gene that was tested (Shapira et al., 1997). The glutathione redox state has been implicated in gene regulation processes in animal and plant cells, as previously reviewed (Noctor et al., 2002). It was also shown to modulate protein activity by formation of intraprotein disulfide bonds (Demple, 1998).

The sensitivity of Rubisco holoenzyme to oxidative stress is well established. Exposure of chloroplasts to oxidizing conditions leads to oxidation of Cys residues and to denaturation of Rubisco, as well as to decreased catalytic activity (Marin-Navarro and Moreno, 2003). Oxidation is also followed by proteolytic cleavage of the LSU to two major polypeptides, sized 37 and 16 kD (Desimone et al., 1996; Ishida et al., 1997). However, the effect of oxidative stress on the assembly process has not been addressed yet. Here we report that light-induced oxidative stress applied to living algal cells confers structural changes in Rubisco and that similar effects can be observed in vitro in the presence of oxidized glutathione (GSSG). We propose here that these structural changes result in exposure of the RNA recognition motif (RRM) that we previously found in the LSU (Yosef et al., 2004). The unchaperoned and exposed RRM will bind any RNA in its vicinity including its own transcript, resulting in the translational arrest of the LSU. Once synthesis of the LSU stops, Rubisco assembly is disrupted in a coordinated manner and the SSU is eliminated by proteolysis.

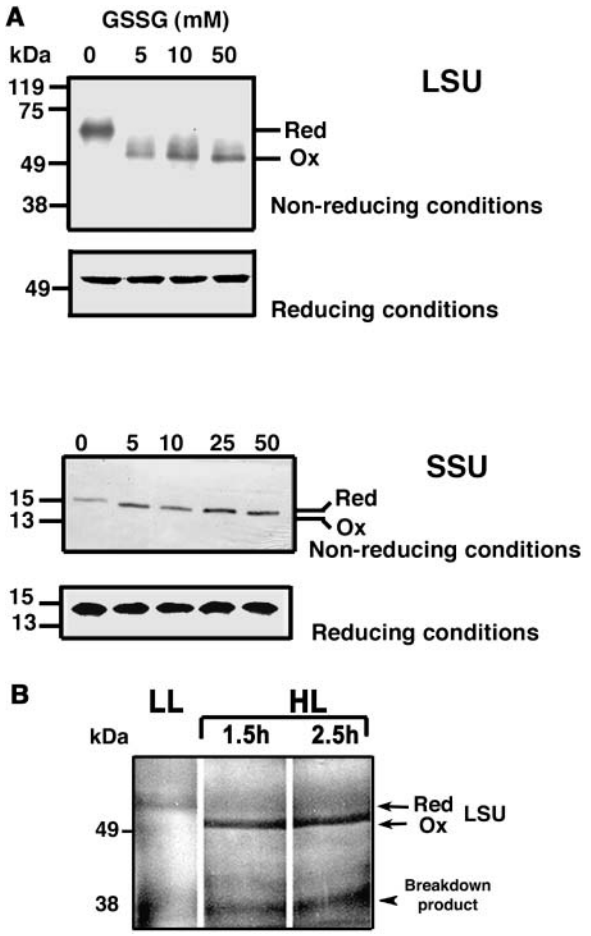

Figure 1. Oxidation of thiol groups on Rubisco subunits by GSSG and by a light stress. A, Purified Rubisco holoenzyme was incubated in vitro with increasing concentrations of GSSG, protonated by precipiation with TCA, resolubilized, and allowed to react with AMS (for the LSU) or iodoacetamide (for the SSU). Following this procedure, the protein complex was loaded on nonreducing SDS-PAGE (top sections), 10% for the LSU and 15% for the SSU. For control, oxidation by GSSG was reversed by the addition of β-mercaptoethanol prior to incubation with the alkyating agent. The samples were then dialyzed against solubilization buffer, the thiol-reacting agent was added, and the samples were separated over a reducing gel (bottom sections). The gels were subjected to western analysis using antibodies against Rubisco. Migration of the reduced (Red) and oxidized (Ox) forms of the protein is marked with arrows. B, *C. reinhardttii* cells were grown at LL and transferred to HL for 1.5 and 2.5 h, then harvested and lysed in the presence of TCA. The precipitated proteins were resolubilized, incubated with AMS, and separated over 10% nonreducing SDS-PAGE as described in A.

RESULTS

Oxidizing Conditions Change the Redox State of Thiol Groups on Rubisco Subunits in Vitro and in Vivo

We examined how oxidative stress affects the thiol groups in Rubisco subunits, both in vitro and in vivo, using thiol-reactive agents, such as 4-acetamido-4′-maleimidyl-stilbene-2,2′-disulfonic acid (AMS) and idoacetamide. After oxidation, the Rubisco holoenzyme was precipitated with TCA that protonates free-thiol groups. The protein was then solubilized and the thiol-reactive agent was added to interact irreversibly with the free-thiol groups on Cys residues. This modification increased the size of the target protein and retarded its migration on nonreducing gels. As suspected, if Rubisco holoenzyme was first oxidized in vitro with increasing concentrations of GSSG and then exposed to the thiol-reacting agent, both the LSU and the SSU migrated faster than the nonoxidized and alkylated Rubisco subunits (Fig. 1A, top sections). This indicated that exposure of Rubisco to GSSG resulted in oxidation of the thiol groups on Cys residues. The observed mobility shift was indicative of the redox state of Cys residues in Rubisco subunits, since it was not observed when the GSSG-mediated oxidation of Rubisco was reversed by the addition of β-mercaptoethanol prior to alkylation with the thiol-reacting agent and separation of the modified proteins on reducing gels (Fig. 1A, bottom sections). Rubisco LSU has 10 Cys residues, at positions 53, 84, 172, 192, 247, 284, 399, 427, 449, and 459 and 2 methyl-Cys at

positions 256 and 369. The Cys pairs 172 to 192 and 449 to 459 can create disulfide bridges within each LSU, while Cys 247 is thought to bridge between 2 adjacent subunits (Taylor et al., 2001; Mizohata et al., 2002). The difference in migration of reduced versus oxidized LSU could not resolve which of the individual residues interacted with AMS, but indicated that the LSU was subject to structural changes under oxidizing conditions.

Each of the 2 Rubisco SSU chains in *C. reinhardtii* contains 4 Cys residues, at positions 41, 65, 82, and 96 (Spreitzer, 2003). A step-wise decrease in migration of the SSU on a nonreducing gel was observed following incubation with iodoacetamide, in response to a gradual increase in GSSG concentrations. This indicated that the four thiol groups were modified in a sequential manner. It was difficult to observe such a gradual change in the LSU, as its structural switch was rather homogeneous. It is possible that a minor size change in the LSU could not be resolved on our gel due to the size of the protein, and therefore the slower migration of the polypeptide could be observed only after a comprehensive modification of multiple thiol groups. Alternatively, the uniform change in the size of the LSU polypeptide could indicate that this subunit was more sensitive to oxidation. Altogether, in the presence of GSSG, thiol groups on both subunits of Rubisco became oxidized, leading to structural changes in the protein.

A photooxidative stress affected the redox state of thiol groups on Rubisco subunits also in vivo. Cells were grown continuously under LL and then shifted to HL for 1.5 and 2.5 h. The LL and HL treated cells were harvested and lysed by TCA to protonate all free-thiol groups. The precipitated proteins were resolubilized and allowed to interact with excess AMS, which alkylated the free-thiol groups of Cys residues. As expected, following the protonation and alkylation procedures, migration of Rubisco LSU on nonreducing gels was faster if it was extracted from HL cells as compared to LL cells. This indicated that upon transfer to HL, thiol groups on cystein residues in Rubisco were oxidized and therefore could not be alkylated by AMS (Fig. 1B). In addition to the change in the redox state, the 37-kD breakdown product was observed at HL, as expected. It was difficult to show the same effect for the SSU of Rubisco in vivo. We assume that under these conditions, SSUs that underwent structural changes were rapidly degraded and therefore could not be detected.

The redox state of thiol groups on the LSU and SSU of Rubisco was modulated by 5 mM GSSG. However, in the presence of H_2O_2, a change in the redox state of thiol groups could be monitored only at very high concentrations (Fig. 2), suggesting that the mere presence of an oxidizing agent did not have a direct effect on the redox state of thiol groups in Rubisco. Most probably, modulation of protein redox state proceeded in vivo via a mediator molecule such as glutathione (Noctor and Foyer, 1998).

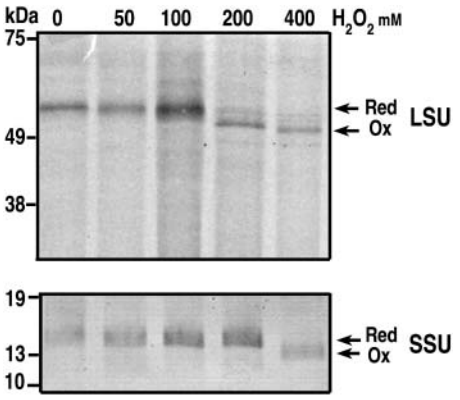

Figure 2. H_2O_2 oxidizes thiol groups on Rubisco subunits only at high concentrations. Purified Rubisco holoenzyme was incubated in vitro with increasing concentrations of H_2O_2 and then allowed to react with AMS. The protein complex was then loaded on nonreducing SDS-PAGE (top sections), 10% for the LSU and 15% for the SSU. The gels were subjected to western analysis using antibodies against Rubisco. Migration of the reduced (Red) and oxidized (Ox) forms of the protein is marked with arrows.

Rubisco Assembly during Oxidative Stress Is Tightly Coordinated with Translation of the LSU, and Assembled Particles Remain Stable during the First Hour at HL

To examine the coordination between the translational arrest of the LSU and cessation of the assembly process, cells were transferred from LL to HL for different time periods (30, 45, and 60 min) and pulse labeled for 3 min with $H_2{}^{35}S0_4$ at the same light conditions. Immediately after the pulse ended, the cells were divided into two parts. One of them was lysed in SDS-sample buffer and separated over SDS-PAGE denaturing gels to follow the labeling of new LSUs (Fig. 3A). The other was disrupted by sonication, and the soluble fraction was separated over native gels to monitor assembly of new Rubisco particles (Fig. 3B). The inhibitory effect of transferring the cells to HL on translation of the LSU and on assembly of the holoenzyme was nonlinear and could be observed only after a lag period of 30 min at HL. After that period of time (45 and 60 min), inhibition of both processes gradually increased with similar kinetics. It appears that the redox-dependent inhibition occurred only after the reactive oxygen species (ROS) reached a certain threshold and that a tight coordination existed between the synthesis of LSU and its assembly into the holoenzyme.

To test the stability of the holoenzyme particles during the first hour after transfer from LL to HL, a pulse-and-chase experiment was performed. Cells grown at LL were transferred to HL for different periods of time (30, 45, and 60 min) and then pulsed during 3 min with $H_2{}^{35}S0_4$, as described above. After the pulse ended, an aliquot was removed from each tube (Fig. 4, P lanes) and the radiolabeled cells were further chased with excess cold H_2S0_4 during 60 min at the same light conditions (C lanes). Following the

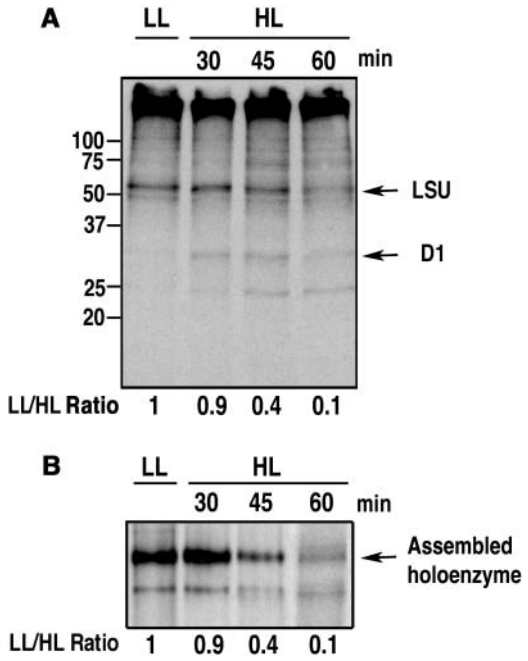

Figure 3. A light-induced oxidative stress causes a coordinated reduction in translation of newly synthesized LSU chains and their assembly. *C. reinhardttii* cells were grown at LL and shifted to HL for 30, 45, and 60 min, and pulse labeled with $H_2{}^{35}SO_4$ for 5 min. The cell culture was divided into two parts and treated as follows: A, Cells were harvested and the proteins extracted by SDS sample buffer. The radiolabeled proteins were separated over SDS-PAGE, and nascent synthesis of the LSU was monitored by autoradiography. The ratio between incorporation of de novo synthesized subunits into holoenzyme complexes at LL and at HL is shown below the autoradiogram (HL/LL Ratio). B, Cells were harvested, disrupted by sonication, and the proteins from the soluble fraction were separated over 5% to 15% native polyacrylamide gels. Assembly of nascent radiolabeled polypeptides was monitored by autoradiography, and the ratio between assembly at LL and HL is shown below the autoradiogram.

chase, the cells were disrupted by sonication, and the soluble fraction that contained the Rubisco holoenzyme was separated over a native gel. The radiolabeled complexes that were isolated immediately after the pulse labeling (P lanes) remained stable during the first hour (C lanes). The breakdown products of the LSU that usually appear in response to severe oxidative-stress conditions (Desimone et al., 1996) could be seen only after longer periods of exposure to HL (data not shown), most probably when ROS levels reach a certain threshold.

Coordinated Expression of Rubisco Subunits under Oxidative Stress Is Achieved by Rapid Degradation of the SSU

Expression of Rubisco subunits should be tightly coordinated, as assembly of the holoenzyme is stoichiometric. Since oxidative stress results in a translational arrest of LSU synthesis and cessation of assembly, we examined how the coordinated expression of the two subunits is achieved. A pulse-and-chase labeling experiment was performed in *C. reinhardtii* cells that were grown in LL and transferred to HL (Fig. 5). Under LL conditions, both LSU and SSU remained stable for over an hour, with no apparent degradation of either subunit. When the cells were pulsed at after 60 min at HL and then chased at the same conditions, the SSU was rapidly degraded within 15 min postlabeling, while the LSU remained relatively stable during the first hour. When the cells were pulsed after 90 min at HL, incorporation into the LSU was very low and the SSU was not detected at all. Thus, the translational arrest of the LSU during oxidative stress is accompanied by a rapid degradation of the SSU.

The *rbcL* Transcript Shifts towards Small Polysomes and Monosomes during Oxidative Stress

Changes in the polysomal distribution of the *rbcL* transcript as a function of illumination were previously demonstrated using Suc gradient analysis (Kim and Mullet, 2003). Here we tested the polysomal distribution of *rbcL* as a function of oxidative stress. We previously showed that exposure of algal cells to methyl viologen (MeV) down-regulated LSU synthesis in a similar manner to that observed during light stress and that this treatment resulted in the increase of ROS and in oxidation of the glutathione pool (Irihimovitch and Shapira, 2000). Since the major portion of the *rbcL* transcript is associated with soluble polysomes, and,

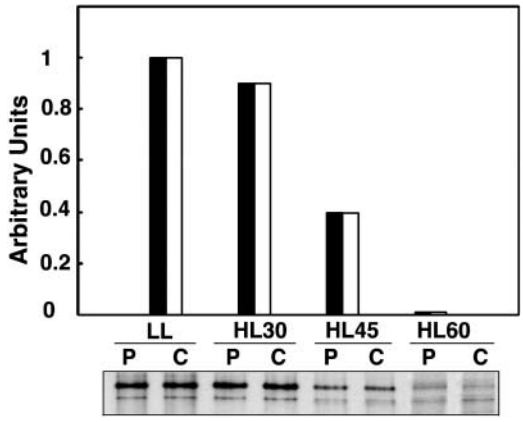

Figure 4. Assembled Rubisco particles are stable during the first hour after transfer from LL to HL. *C. reinhardttii* cells were grown at LL and shifted to HL for 30, 45, and 60 min, and pulse labeled with $H_2{}^{35}SO_4$ for 3 min. The cells were then chased by the addition of cold 10 mM Na_2SO_4 and further incubated for an additional hour under the same light conditions. Cell aliquots were removed after the pulse labeling, before (P) and after (C) the chases, to monitor the stability of newly assembled Rubisco complexes under the conditions tested. The cells from each aliquot were disrupted by sonication, and proteins from the soluble fraction were analyzed over 5% to 15% native polyacrylamide gels. Assembly of nascent radiolabeled polypeptides was monitored by autoradiography, and the ratio between the level of incorporation into Rubisco complexes in the pulsed (black columns) and in the chased (white columns) cells is shown by a histogram.

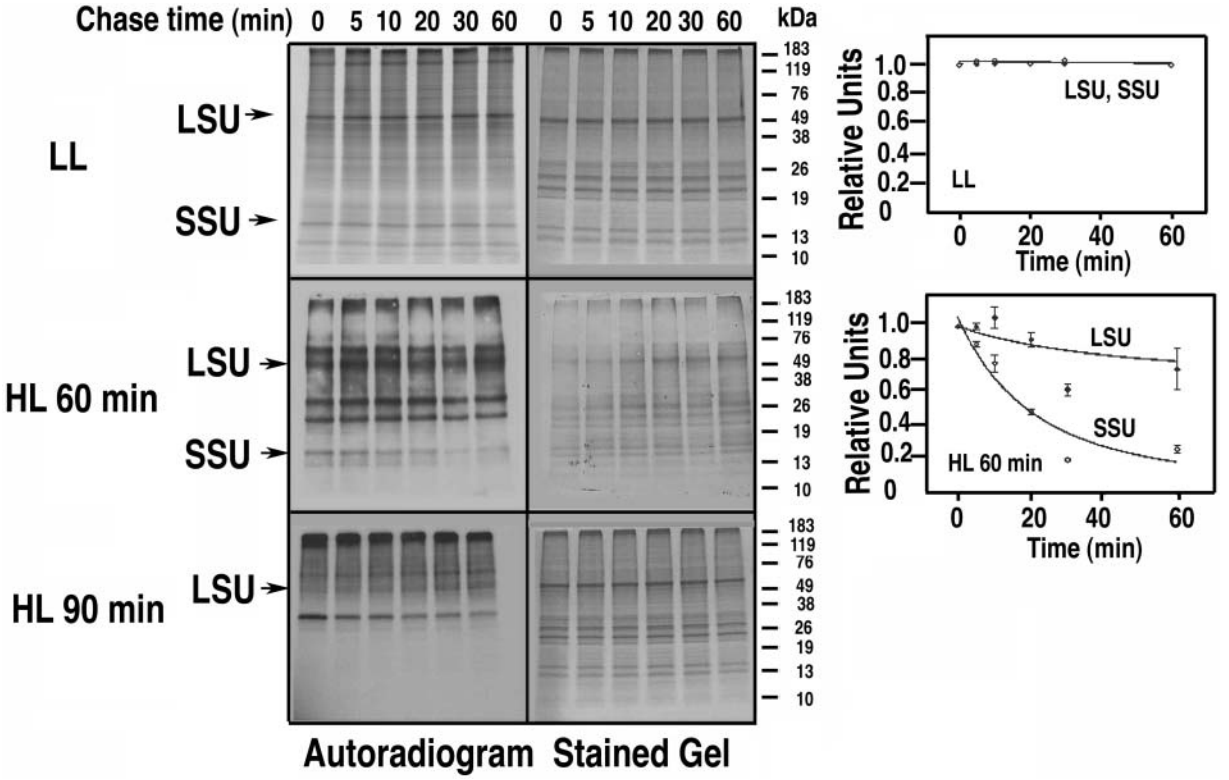

Figure 5. Pulse-and-chase labeling of Rubisco subunits at LL and during a light-induced stress. *C. reinhardtii* cells were grown at LL and shifted to HL for 60 and 90 min. The cultures were then pulsed with $H_2{}^{35}SO_4$ for 3 min and chased by the addition of cold Na_2SO_4 10 mM. Samples were removed from the chased cultures at different time points for 1 h at the corresponding light conditions, and total proteins were extracted for analysis on 10% to 20% SDS-PAGE. Migration of the Rubisco subunits was verified by parallel western analysis. The decay rates of LSU (♦) and SSU (◇) polypeptides were determined by Phosphorimager analysis, and the results are presented in the accompanying graphs.

accordingly, the protein product of *rbcL* is found predominantly in the chloroplast-soluble phase (Klein et al., 1988; Kim and Mullet, 2003), our analysis focused on the soluble polysome fraction. Polysomes from control cells and from cells that were exposed to MeV were separated using Suc gradients, and RNA was extracted from the different fractions. Northern analysis revealed that the majority of the *rbcL* transcript was associated with the polysomal fraction, in accordance with previous reports (Klein et al., 1988). In MeV treated cells, the *rbcL* transcript was still associated with polysomes; however, it shifted toward fractions containing smaller polysomes and monosomes (fractions 17–21, Fig. 6). This result suggests that the inactivation of *rbcL* translation as a result of oxidative stress occurs, most probably, at the initial stage of translation elongation. While at normal conditions only a small fraction of the *rbcL* transcript was associated with monosomes or small polysomes (Fig. 5A, fraction 21; 13.8%), under conditions of oxidative stress, the portion of the *rbcL* transcript that was associated with this fraction increased 2-fold (Fig. 5B, fraction 21; 25.8%). The amount of the *rbcL* transcript that was associated with the small ribosomal subunit remained relatively unchanged (Fig. 5, A and B, fraction 23 in each section).

DISCUSSION

We show here that inhibition of LSU translation and assembly of the holoenzyme complex are tightly coordinated. The inhibition of both processes is nonlinear, since it can be monitored only after a lag time of 30 min at HL. After this time point, the translational arrest gradually increases along with a cessation of the assembly process (Fig. 3), suggesting that a certain threshold of ROS must be reached before inhibition occurs. Concomitantly, the SSU is removed by rapid degradation of nonassembled molecules. Expression of Rubisco subunits is tightly coordinated. It was previously reported that silencing of SSU expression by introducing antisense RNA (Rodermel et al., 1996) or by a gene knockout (Khrebtukova and Spreitzer, 1996) prevented the accumulation of the LSU. Similarly, when translation of Rubisco LSU was inhibited by a nonsense mutation or by chloramphenicol, the SSU was rapidly degraded, indicating that it cannot accumulate in its free and unassembled form (Schmidt and Mishkind, 1983; Spreitzer et al., 1985).

Oxidative stress confers structural alterations on both subunits of Rubisco. This is reflected in experiments using AMS and iodoacetamide, thiol-reactive agents that bind reduced-thiol groups on both

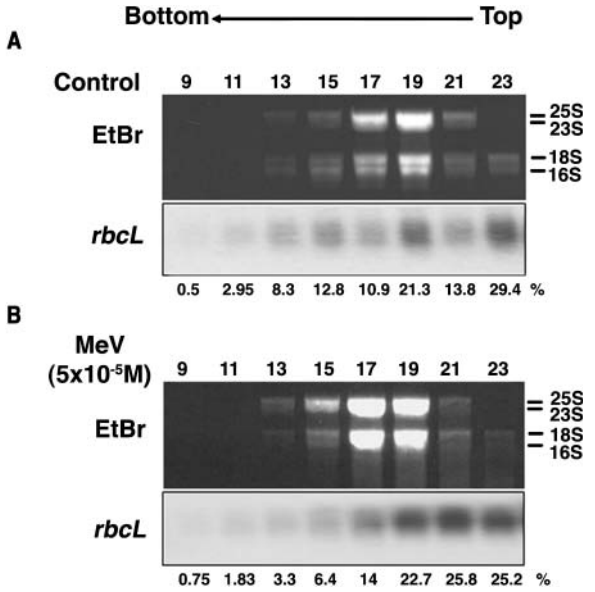

Figure 6. Polysome association of the *rbcL* transcript at normal and oxidizing conditions. Polysomes were prepared as described from cells grown at normal conditions, and after their exposure to MeV 10^{-5} M, for 1 h. The RNA content (ethidium bromide staining) is shown at the top sections, and hybridization to the *rbcL* probe is shown at the bottom section. The polysome association pattern of the *rbcL* transcript is shown for cells grown at LL (A) and for cells treated with MeV (B). The relative intensity of hybridization to the *rbcL* probe is shown below the autoradiograms.

subunits and retard their migration in nonreducing gels. This binding can be inhibited by the addition of GSSG in experiments carried out in vitro. For the SSU, a gradual oxidation of the four thiol groups is observed in response to an incremental increase in GSSG concentration. Indeed, the Cys residues vary in their surface exposure, possibly affecting their accessibility to the oxidizing agent GSSG. Whereas Cys-65 is exposed to the surface and Cys-41 is found on an external loop, Cys-83 and Cys-96 are buried within the protein. It can be assumed that the exposed Cys residues will be oxidized at lower concentrations of GSSG, as compared to their internal counterparts. A similar analysis of the LSU showed that this polypeptide was more sensitive to oxidation by GSSG, and a maximal effect on its migration was observed already at the lower concentration of GSSG.

It is interesting to note that an oxidizing agent such as H_2O_2 confers structural changes in vitro only when present at a relatively high concentration (>100 mM), unlike GSSG, which affects the redox of thiol groups on Rubisco already at moderate concentrations (5 mM). Thus, H_2O_2 by itself most probably does not act directly on Rubisco thiol groups and requires a mediator molecule, such as glutathione. Indeed, we previously showed that elevation of ROS during transfer of *C. reinhardtii* from LL to HL is accompanied by oxidation of the glutathione pool, and these changes correlate with the temporal arrest of Rubisco LSU synthesis (Irihimovitch and Shapira, 2000).

Applying an oxidative stress in vivo by shifting the cells from LL to HL had a similar effect on the redox state of thiol groups in Rubisco. Due to the magnitude of the change observed by western analysis, the oxidizing environment most probably affected the majority of the LSU polypeptide molecules in the cell. We assume that this change precedes the breakdown of LSU, and, moreover, it is possible that the structural changes are not restricted only to the holoenzyme, but they also occur in preassembled de novo-synthesized polypeptides.

It was previously shown that a severe oxidative stress leads to the proteolytic breakdown of Rubisco in intact chloroplasts (Desimone et al., 1996; Ishida et al., 1997), which is dependent on ATP hydrolysis (Desimone et al., 1998). By separating protein extracts of metabolically labeled cells on native gels and by monitoring the assembly of newly synthesized subunits, we show that light-induced oxidative stress interferes with Rubisco assembly. Based on these results, we hypothesize that oxidation of thiol groups on Rubisco subunits confers structural changes that disrupt the process of Rubisco assembly, in parallel to the LSU translation. It is important to note, however, that once assembly occurred, the holoenzyme remained relatively stable during the first hour after transfer to HL (Fig. 4), and its steady-state level at HL went down only after 2 h following the light shift (data not shown).

We recently showed that the amino-terminal domain of the LSU has a structural resemblance to the RRM, typical of many RNA-binding proteins, such as the U1A splicing factor (Yosef et al., 2004). We also noted that the RNA-binding activity of the LSU was observed exclusively under oxidizing conditions, either when it was part of the holoenzyme or when the complete LSU chain was expressed in bacteria. The RRM-like domain is buried within the LSU and becomes exposed to the surface only under oxidizing conditions. These confer structural alterations that are mediated by changes in the redox state of the thiol groups. The RNA-binding activity of the LSU, measured by UV cross-linking experiments, is sequence independent. However, we propose that this activity could be involved in an autoregulatory loop that specifically controls translation of the LSU. Assembly of Rubisco initiates with the association between de novo-synthesized polypeptide chains and molecular chaperonins (Gutteridge and Gatenby, 1995; Hartl, 1996). In addition to cpn60 (Gutteridge and Gatenby, 1995), a DnaJ-related protein is required for accumulation of Rubisco in bundle sheath cells of maize (Brutnell et al., 1999). Indeed, a microarray analysis of pooled expressed sequence tag libraries, prepared from *C. reinhardtii* cells that were exposed to HL (1,100 μmol photon m^{-2} s^{-1}) or deprived of oxygen, iron, and copper for various time periods (Shrager et al., 2003), indicated that expression of DnaJ- and DnaK-like

proteins increased when exposed to these conditions. Involvement of DnaK and DnaJ proteins was indicated in *E. coli* expressing bacterial hexameric Rubisco (Checa and Viale, 1997). Recently, a redox-regulated chaperone network was identified in *E. coli* and reconstituted in vitro, involving Hsp33, DnaK, DnaJ, and GrpE (Hoffmann et al., 2004). We therefore propose that oxidative stress causes a conformational change of the LSU already during its synthesis. This change could affect its ability to interact with chaperone proteins, and thus, the polypeptide chain would not be able to fold properly. Under these conditions, the amino-terminal domain that contains an RRM motif is exposed and is therefore capable of binding any RNA found in its vicinity, namely the *rbcL* transcript. The SSU most probably does not function as the original redox-sensing molecule, since its elimination lagged after the translation arrest of the LSU. SSU degradation could be monitored only after the cells were exposed to HL during 60 min, while synthesis of LSU and assembly had already reduced 45 min after the transfer to HL. This and the tight coordination between LSU synthesis and Rubisco assembly suggest that light-induced redox regulation of Rubisco formation is initially sensed by the LSU.

Further support of this mechanism is obtained from analyzing the profile of polysomes that are associated with the *rbcL* transcript, using Suc gradients. Indeed, the *rbcL* transcript remains associated with polysomes even after translation of LSU stops, although upon transfer of the cells from LL to HL, the *rbcL* RNA shifts toward smaller polysomes and monosomes. The continuous association of the *rbcL* transcript with the polysomal fraction suggests that the arrest in translation occurs at a postinitiation stage. If exposure of the RRM motif in a newly synthesized chain and its interaction with the *rbcL* transcript causes the ribosome to pause at an early stage of elongation, then new ribosomes cannot be added, causing the observed shift toward smaller polysomes and monosomes. A similar mechanism that controls ribosome loading was reported in other systems for hydroxyperoxide glutathione peroxidase in eukaryotes (Fletcher et al., 2001). Studies on translation of the *rbcL* RNA in barley (*Hordeum vulgare*) during dark-to-light transition also suggest that translation regulation of *rbcL* occurs at the elongation stage. Although exposure of barley seedlings to light for 16 h increases the amount of polysome-associated *rbcL* mRNA, the abundance of translation initiation complexes bound to the *rbcL* message remained unchanged, suggesting that translation regulation may be controlled at the elongation stage (Klein et al., 1988; Kim and Mullet, 2003). Studies on chloroplast complexes that are composed of multiple subunits, such as cytochrome *f* and PSI, indicated that coordinated expression of the different subunits is obtained by control of translation initiation and by enhanced proteolysis of unassembled polypeptides (Choquet et al., 1998; Wollman et al., 1999). However, it appears that in the chloroplast, biogenesis of different complexes may pursue alternative regulatory pathways, since we show here for Rubisco that, under conditions of oxidative stress, control of its LSU synthesis occurs after translation initiation has occurred.

Whether the regulatory mechanism in *C. reinhardtii* is also conserved in land plants is yet to be shown. However, preliminary experiments (data not shown) using Rubisco holoenzyme that was purified from tobacco (*Nicotiana tabacum*) and spinach (*Spinacia oleracea*) show RNA-binding activity in vitro in the presence of GSSG.

CONCLUSION

Here we examine the inhibitory effect of oxidative stress on Rubisco assembly and on expression of its subunits. Oxidative stress affects thiol groups of Rubisco subunits, as observed in vivo during light-induced stress and in vitro in the presence of GSSG. We propose that the redox of the thiol groups causes structural changes of newly synthesized polypeptide chains, resulting in the exposure of an RRM domain found in the amino terminus of Rubisco LSU and in binding of the *rbcL* transcript while still on the polysomes. Thus, translation of the LSU is inhibited almost completely during oxidative stress, most probably by an autoregulatory loop. Assembly of the holoenzyme therefore halts, and the unassembled SSU polypeptides are rapidly degraded. Upon recovery from oxidative stress, synthesis of the LSU and assembly of both subunits resume.

MATERIALS AND METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii wild-type strain CC-125, obtained from the Chlamydomonas Genetics Center, Duke University at Durham, NC, was used in all experiments. Cultures (300 mL) in high-salt-reduced sulfate (HSRS; Schmidt et al., 1985) were grown at 25°C with 5% CO_2 bubbling and constant rotary shaking. Cultures were illuminated with LL (70 μmol m^{-2} s^{-1}) using cool-white fluorescent lamp. LL-grown cells were adapted for low irradiance and were not permitted to attain densities greater than 0.2 to 0.3 A_{750}.

Measuring the in Vitro Redox State of Rubisco Subunits Exposed to GSSG and H_2O_2

The effect of GSSG or H_2O_2 on the oxidation state of Rubisco subunits was examined by the use of thiol-reacting agents. The free-thiol groups on Cys residues were modified by iodoacetamide for the SSU and by AMS for the LSU. Briefly, purified Rubisco (100 μL; 0.2 mg/mL) was subjected to various concentrations of GSSG and H_2O_2 for 1 h at room temperature. The protein was then precipitated by the addition of TCA to a final concentration of 20% for 30 min to protonate all free thiols. Protein precipitates were collected by centrifugation at 13,000 rpm for 10 min, washed with acetone, and dissolved in freshly prepared solution containing 1% SDS, 100 mM Tris-HCl, pH 8, 1 mM EDTA, and a cocktail of protease inhibitors. The thiol-reacting agent was added to a final concentration of 20 mM for AMS or 75 mM for iodoacetamide, and the mixture was incubated for 1 h at room temperature. Protein samples were then separated over nonreducing gels, 15% (for SSU) or 10% (for LSU) SDS-PAGE (30:0.4 acrylamide:bisacrylamide), lacking any reducing agent, and subjected

to western analysis using anti-Rubisco antibodies (1:4,000). In control samples, oxidation by GSSG was reversed prior to the incubation with iaodoacetamide or AMS, by the addition of β-mercaptoethanol to a final concentration of 2% and dialysis against the solubilization buffer. The thiol-reacting agent was then added, and the samples were separated over reducing gels.

Measuring the in Vivo Redox State of Rubisco Subunits

The in vivo redox state of Rubisco subunits was determined as described (Vestweber and Schatz, 1988) using AMS (Molecular Probes, Eugene, OR). Cells were grown under LL in HSRS until their biomass reached A_{750} 0.1 to 0.2, harvested after centrifugation, and resuspended at 0.5 A_{750} in high-salt medium lacking sulfate (HS-S) for 1.5 h under LL conditions. Cell aliquots (4 mL) were placed in 30-mL Corex tubes containing magnetic stirring bars and illuminated at LL or HL for 1.5 to 2.5 h. Whole-cell proteins from 3-mL samples were precipitated by TCA at a final concentration of 20%. Protein precipitates were collected after centrifugation, washed with acetone, and dissolved in a freshly prepared solution containing 20 mM AMS (Molecular Probes), 1% SDS, 100 mM Tris-HCl, pH 8, 1 mM EDTA, and protease inhibitors cocktail (Sigma-Aldrich, St. Louis). Protein extracts were separated over nonreducing 10% SDS-PAGE (30:0.4 acrylamide:bisacrylamide) lacking any reducing agent and subjected to western analysis using anti Rubisco antibodies (1:4,000).

In Vivo Pulse-and-Chase Labeling of Chloroplast Proteins

In vivo pulse-and-chase labeling of plastid and nuclear proteins with $H_2{}^{35}SO_4$ was preformed essentially as described (Schmidt and Mishkind, 1983) with the following modification. Cells were grown under LL in HSRS until their biomass A_{750} reached 0.1 to 0.2. The cells were then harvested following centrifugation for 5 min (4,500*g*, 23°C) and resuspended at 0.5 A_{750} in HS-S (Schmidt et al., 1985) containing 10 mM bicarbonate to ensure carbon availability for photosynthesis. The cells were equilibrated for 1.5 h under LL in HS-S. Aliquots (4 mL) were placed in 30-mL Corex tubes containing magnetic stirring bars and illuminated at LL or HL (700 μmol m^{-2} s^{-1}) for a designated time period. For the pulse labeling, $H_2{}^{35}SO_4$ (carrier free, New England Nuclear, 250 μCi) was added for 3 min, and chase periods initiated upon addition of 0.01 vol of 1 M Na_2SO_4 (40 μL, to reach a final concentration of 10 mM). Aliquots of 600 μL were removed after 0, 5, 10, 20, 30, and 60 min into 5.4 mL of 100% ice-cold acetone, incubated on ice for 1 h, and centrifuged for 10 min. The protein pellets were dried and resuspended in 70 μL distilled, deionized water, followed by the addition of 70 μL denaturing solution (4%SDS, 5 mM EDTA, 40 mM Tris-HCl, pH 7.4), and the mixtures were briefly vortexed and boiled for 10 min. Incorporation of the radiolabel was measured by TCA precipitation. Equal amounts of labeled proteins were separated over 10% to 20% gradient SDS-polyacrylamide gels. The gels were stained by Coomassie Blue, dried, and subjected to autoradiography or analyzed by Fuji phosphorimager (Fugi Photo Film, Tokyo).

Rubisco Purification

Wild-type *C. reinhardtii* cells (CC-125) were grown in a Tris-acetate phosphate medium (3 L) and harvested to yield 8 g fresh weight. The cell pellet was resuspended in 30 mL buffer containing 50 mM Tris-HCl, pH 8, 10 mM Mg_2Cl, 10 mM $NaHCO_3$, 10 mM dithiothreitol (DTT), 1 mM EDTA, and a cocktail of protease inhibitors (Sigma-Aldrich). The cells were disrupted by a triple passage through a French Pressure Cell Press (Spectronic Instruments, Rochester, NY) at 4,000 psi and centrifuged for 10 min at 20,000*g* (Sorvall SS-34 rotor, 10,000 rpm; Sorvall Products, Newtown, CT). The supernatant was collected and further centrifuged at 200,000*g* for 1 h at 4°C (Beckman TI50 rotor, 50,000 rpm; Beckman Instruments, Fullerton, CA). The protein was precipitated by a 25% to 50% ammonium sulfate cut of the 200,000*g* supernatant, and the pellet was resuspended and dialyzed against a buffer containing 50 mM Tris-HCl, pH 8, 10 mM Mg_2Cl, 10 mM $NaHCO_3$, 10 mM DTT, and 1 mM EDTA. The dialyzed solution was loaded on a linear Suc gradient (10%–30%) prepared in dialysis buffer. The gradient was centrifuged for 16 h at 4°C at 164,000*g* (Beckman SW40, at 40,000 rpm) and fractions (1 mL) were collected. Migration of Rubisco holoenzyme was monitored by western analysis of samples removed from each fraction.

Antisera

Polyclonal rabbit antisera used in this study were directed against the Rubisco holoenzyme from tobacco (*Nicotiana tabacum*).

Separation of Pulse-Labeled Rubisco over Native Gels and Western Blotting

Cells were grown at LL conditions and transferred to HL for time periods of 30, 45, and 60 min. Following this treatment, the cells were pulse labeled for 3 min and chased, as described above. Samples (2 mL) were removed at time 0 and after a chase of 60 min, centrifuged, washed once with HSRS, and resuspended in 500 μL of sonication buffer (2 mM DTT, 10 mM $MgCl_2$, 2 mM $NaHCO_3$, and 50 mM Tris-HCl, pH 8.0). Samples were immediately sonicated twice using a microtip of XL 2020 MISONIX sonicator at 0°C. Sonication was performed by applying pulses of 20 s at intervals of 20 s. The disrupted cells were then centrifuged for 10 min at 4°C (20,000*g*), and supernatants containing the soluble proteins were collected. Incorporation of the radiolabel was measured by TCA precipitation. The assembled Rubisco holoenzyme was analyzed using nondenaturing 5% to 15% gradient polyacrylamide gels separated at 4°C in the standard Tris-Gly buffer lacking SDS, at 100 mV for 5 h. For western blotting, the gels were first soaked in transfer buffer containing 10% SDS for 10 min, and blotting time was extended (×2).

Fractionation of Soluble Polysomes on Suc Density Gradients

A 300-mL culture was grown at LL until log phase (OD_{750} 0.2–0.3). To produce an oxidative stress, MeV was added at a concentration of 10^{-5} M for 1 h at LL. Cycloheximide and chloramphenicol were added to concentrations of 10 μg/mL and 100 μg/mL, respectively, for 5 min. The cells were harvested and resuspended in a buffer containing 25 mM Tris-HCl, pH 8, 25 mM MgCl, 25 mM KCl, 5 mM DTT, 10 μg/mL cycloheximide, 100 μg/mL chloramphenicol, a proteinase inhibitor cocktail (Sigma-Aldrich, according to the manufacturer), and 1 mg/mL Heparin. The cells were disrupted three times in a French Pressure Cell Press (at 5,000 psi). The cell debris and insoluble membranes were removed by centrifugation at 18,000*g* for 40 min at 4°C. The supernatant (5 mL) was layered over a 10%-to-40% Suc gradient prepared in the resuspention buffer, and the gradients were centrifuged at 120,000*g* for 4.5 h at 4°C in a SW28.1 rotor (Beckman). RNA was isolated from the fractions using phenol/chloroform extraction followed by ethanol precipitation. The RNA was separated using 1% agarose gels containing formaldehyde, blotted, and hybridized with a 1.5-kb radioactive fragment containing the entire *rbcL* gene, amplified by PCR from p266 (provided by the Chlamydomonas Genetics Center, Duke University, Durham, NC).

ACKNOWLEDGMENTS

We thank T.J. Andrews from the Australian National University, Canberra, Australia for antibodies against Rubisco, and T. Trebitsh from the Ben-Gurion University for helpful discussions.

Received November 11, 2004; returned for revision December 2, 2004; accepted December 8, 2004.

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