RNA Binding Activity of the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Large Subunit from *Chlamydomonas reinhardtii*

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Transfer of the green algae *Chlamydomonas reinhardtii* from low light to high light generated an oxidative stress that led to a dramatic arrest in the synthesis of the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The transgenic arrest correlated with transient changes in the intracellular levels of reactive oxygen species and with shifting the glutathione pool toward its oxidized form (Irihimovitch, V., and Shapira, M. (2000) *J. Biol. Chem.* 275, 16289–16295). Here we examined how the redox potential of glutathione affected the RNA-protein interactions with the 5′-untranslated region of *rbcL*. This RNA region specifically binds a group of proteins with molecular masses of 81, 62, 51, and 47 kDa in UV-cross-linking experiments under reducing conditions. Binding of these proteins was interrupted by exposure to oxidizing conditions (GSSG), and a new protein of 55 kDa was shown to interact with the RNA. The 55-kDa protein comigrated with Rubisco LSU in one- and two-dimensional gels, and its RNA binding activity was further verified by using the purified protein in UV-cross-linking experiments under oxidizing conditions. However, the LSU of purified and oxidized Rubisco bound to RNA in a sequence-independent manner. A remarkable structural similarity was found between the amino-terminal domain of Rubisco LSU in *C. reinhardtii* and the RNA binding domain, a highly prevailing motif among RNA-binding proteins. It appears from the crystal structure of Rubisco that the amino terminus of LSU is buried within the holoenzyme. We propose that under oxidizing conditions it is exposed to the surface and can, therefore, bind RNA. Accordingly, a recombinant form of the polypeptide domain that corresponds to the amino terminus of LSU was found to bind RNA in vitro with or without GSSG.

When plants and algae absorb light energy that exceeds the level of electron carrier saturation they generate reactive oxygen species (ROS), that cause a variety of cellular and molecular damage. This phenomenon is referred to as photoinhibition and is common to all photosynthetic organisms (1–3). Recovery from photoinhibition can be achieved by decreasing the chlorophyll content and by activating a variety of antioxidative pathways that involve ascorbate and glutathione (4, 5). Ribulose-1,5-bisphosphate carboxylase (Rubisco) is the key enzyme in photosynthetic carbon assimilation. In *Chlamydomonas reinhardtii* and in land plants the enzyme is composed of eight large subunits (LSU) encoded by the chloroplast *rbcL* gene and eight small subunits encoded by the nuclear *rbcS* gene family. Assembly of the holoenzyme is mediated by the chloroplast chaperonins cpn60 and cpn10 (6, 7). We previously showed that transfer of the green algae *C. reinhardtii* from low light (70 μmol m⁻² s⁻¹) to high light (700 μmol m⁻² s⁻¹) generates an oxidative stress that leads to photoinhibition and a dramatic arrest in the synthesis of the LSU of Rubisco (8). These light-induced effects were found to be transient, with cell recovery taking place within 6–12 h once chlorophyll levels were reduced and ROS levels were decreased. It was further found that translation of Rubisco LSU varies with the changes in ROS production and correlates with alterations in the ratio between oxidized and reduced glutathione. Upon transfer to high light the glutathione pool shifts to its oxidized form, and LSU synthesis stops almost completely. When the cells recover from light stress, the glutathione pool shifts back to its reduced form, and LSU translation resumes (9).

Rubisco holoenzyme is highly susceptible to oxidative stress in vivo. Excess ROS caused a rapid translocation of the soluble enzyme complex into the chloroplast membrane and the formation of intermolecular cross-linking between the large subunits via disulfide bonds (10). Furthermore, oxidative stress can cause direct fragmentation of Rubisco LSU at Gly-329 into 37- and 16-kDa polypeptides in illuminated intact chloroplasts in chloroplast extracts and in its purified form when exposed to a hydroxyl radical-generating system (11).

Translational regulation allows plants to respond quickly to environmental changes such as light intensities and is, therefore, a predominant mechanism in chloroplasts. Upstream UTRs are expected to play a key role in translation of chloroplast genes via interaction with regulatory proteins. A group of RNA binding (RB) proteins with molecular masses of 60, 55, 47, and 38 kDa assemble on the 5′-UTR of the *psbA* RNA (12). RB47 shows a high homology with the eukaryotic poly(A) binding protein (13), and RB60 shows high homology to protein disulfide isomerase (14, 15). Binding of the protein complex is mediated by specific thiol-containing proteins, and light-induced reduction of thioredoxin enhances this binding. It was proposed that RB60 serves as a redox sensor that activates

RNA binding domain; LSU, large subunit; UTR, untranslated region; DTT, dithiothreitol; aa, amino acids.

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The abbreviations used are: ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RB, RNA binding; EMSA, electrophoretic mobility shift assays; SK, pBluescript; RBB,
binding of RB47 (16). Proteins with similar molecular masses assemble on the 5′-UTR of pshC and other chloroplast leader sequences (17, 18), although it is possible that different RNA-binding proteins of size 47 kDa have altered target specificities (19).

In an attempt to examine the mechanism that underlies the unique pattern of regulation observed for Rubisco LSU, we examined how the redox state of RNA-binding proteins affects their interaction with the rbcL leader. We show that the interaction between RNA-binding proteins and the rbcL leader is interrupted by oxidative stress and that Rubisco LSU can bind RNA in its oxidized form, although in a nonspecific manner.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—*C. reinhardtii* wild type CC-125 cells were grown in high salt reduced sulfate medium with bubbling of 5% CO₂ and constant rotary shaking at 25 °C. Cultures were illuminated with medium light (150 μmol m⁻² s⁻¹) using cool white fluorescent lamps. The photosynthesis-deficient mutant CC-2653 has an amber mutation at the 5′ end that terminates translation of Rubisco LSU at position 85 (20) and was grown on Tris acetate phosphate medium at 25 °C under dark conditions. For Rubisco purification CC-125 cells were grown in Tris acetate phosphate medium under low light conditions (70 μmol m⁻² s⁻¹).

**Plasmids Used for in Vitro RNA Synthesis**—The 5′-UTR of rbcL from *C. reinhardtii* was cloned from P-266, a plasmid that contains the 4-kilobase EcoRI-BamHI fragment of chloroplast DNA (21). An EcoRI-XhoI fragment of the insert was inserted into pBlueScript at the EcoRI-ClaI site, resulting in pVII. The complete 5′-UTR of rbcL (between positions −93 to +24 relative to the ATG start codon) was amplified from pVII using the primers 5′-TAAATGTTATTTTTTTCAACAACT3′ (forward) and 5′-TCTTAGTTCTGTGTTGGAACCAT3′ (reverse). The resulting PCR fragment was cloned into the pGEM-T vector (Promega), and sequences between the PsI and NsiI sites were removed in pVIII. The plasmid encoding rbcL was linearized by NcoI and NotI for synthesis of the sense and antisense strands, respectively.

**In vitro transcription of the 5′-UTRs derived from pshA, atpB chloroplast genes, and α-tubulin (a nuclear gene) was performed from plasmids D1-HA, P-419, and P-654, respectively. Plasmid D1-HA was a generous gift of A. Danon; plasmids P-419 and P-654 were obtained from the Chlamydomonas Center at Duke University. Plasmid D1-HA was linearized with EcoRI, and P-419 and P-654 were linearized with BamHI. In vitro synthesis of the sense strands derived from the corresponding 5′-UTRs were performed with T7 RNA polymerase. Synthesis of a non-related RNA fragment was performed using T7 RNA polymerase along with 0.2 units of RNasin (Promega) as a template. The plasmid was linearized by NotI, and RNA was synthesized with T7 RNA polymerase.

**Preparation of Protein Extracts**—*C. reinhardtii* wild type CC-125 cells were grown at medium light, and protein extracts were prepared essentially as described previously (12, 17). Cells (3 × 1 liter) were grown in high salt reduced sulfate and harvested at a concentration of 5 × 10⁶ cells/ml. The cells were frozen in liquid nitrogen and stored at −70 °C until use. Cell pellets (8 g fresh weight) were thawed in 25 ml of low salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂, and 10 mM DTT) in the presence of protease inhibitors (2 μg/ml aprotonin, 10 μg/ml benzamidine, 5 μg/ml leupeptin, and 76 μg/ml phenylmethylsulfonyl fluoride). The cells were disrupted by passage through a French press (Sim-Amico, Spectronic Instruments) at 4000 p.s.i. The broken cells were centrifuged for 10 min at 20,000 × g (SS-34 rotor, 10,000 rpm in an RC-2 Sorvall). The supernatants were collected and further centrifuged at 200,000 × g for 1 h at 4 °C (Ti50 rotor, 50,000 rpm in an L8–85 Beckman ultracentrifuge). The 200,000 × g supernatant was collected and applied immediately onto a 5-ml heparin-Agarose column (Amersham Biosciences) at a flow rate of 1 ml/min. The column was first preswelled with 3 volumes of high salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 10 mM DTT, and 2 mM potassium acetate) and equilibrated with extraction buffer containing (20 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 0.1 mM EDTA, and 2 mM DTT). The bound proteins were eluted with a continuous gradient of potassium acetate concentrations (0–1.6 M) in low salt buffer. Fractions (500 μl) were collected, dialyzed against dialysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM potassium acetate, 0.2 mM EDTA, 2 mM DTT, and 20% glycerol), and stored at −70 °C. The fractions were analyzed by SDS-PAGE (12%), and their protein content was evaluated by Coomassie Blue staining.

**In Vitro RNA Synthesis**—Radiolabeled RNA transcripts (described above) were synthesized in vitro using 0.5–1 μg of DNA in 40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 12.5 mM NaCl, 10 mM DTT, 20 units of RNasin, 0.5 mM ATP, GTP, and CTP, 12 μM UTP, 50 μCi of [32P]UTP (800 Ci/mmol, Amersham Biosciences), and 20 units of SP6 (Roche Applied Science) or T7 (Promega) RNA polymerase in a reaction volume of 20 μl. The reactions were performed at 37 °C for 40 min followed by the addition of 1 unit of DNase I (RNase-free, Promega) and were then incubated for an additional 30 min at 37 °C. The labeled RNAs were separated from the unincorporated ribonucleotides on a spun-down mini-column of Sephadex G-50 in double-distilled H₂O. Under these conditions transcripts were labeled to specific activities that ranged between 5 × 10⁶ and 2 × 10⁷ cpm/μg of RNA. Unlabeled transcripts that were used for competition assays were synthesized as described above, except that the reactions were scaled up to 100 μl, and all the four ribonucleotides were included at equal concentrations (0.5 mM). RNA products were analyzed on 7 m urea, 6% polyacrylamide gels to verify production of a single transcript and to evaluate its size and concentration.

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays (EMSA) were performed essentially as described previously (17). Samples from the heparin-Actigel column (2 μl containing approximately 9 μg of protein) were preincubated for 10 min at room temperature with 5 units of RNasin (Promega) in 3 mM MgCl₂ in a total volume of 5 μl. The mixtures were then added to the RNA probes (0.1 μg of Escherichia coli DNA (49) in a presence of 0.5 units of Escherichia coli T4 DNA ligase) in a final volume of 15 μl. After an incubation of 15 min at room temperature, 2 μl of loading buffer (0.25 μg/ml xylene cyanol, 0.25 μg/ml bromphenol blue, and 6% (v/v) glycerol) were added, and the reactions were separated on a native 5% polyacrylamide gel (acrylamide:bisacrylamide 49:1) in 1× TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8). Running conditions were 25 mA for 2–3 h. The gels were then fixed in a solution of 20% methanol and 10% acetic acid, dried, and subjected to autoradiography. In competition experiments unlabeled RNA transcripts were added in varying amounts of mass excess and preincubated with the protein samples before the addition of the radiolabeled probe.

**UV Cross-linking Assays**—Binding assays were performed as previously described (17). Samples of proteins eluted from the heparin-Actigel column (2 μl containing approximately 9 μg of protein), purified Rubisco (25 ng), or the recombinant polypeptides that corresponded to Rubisco LSU (amino acids 1–475) or its sub-fragments (amino acids 1–150 and 151–475) after purification over a nicked nitroliciatriacetic acid column (25 ng) were preincubated for 10 min at room temperature with 0.5 units of RNasin (Promega) in 3 mM MgCl₂ in a volume of 5 μl. Radiolabeled RNA (100,000 cpm) was then added to the protein solution along with 0.3 μg of *E. coli* tRNA (Sigma) in a final volume of 15 μl. After 15 min of incubation at room temperature the binding reactions were placed on ice and cross-linked by UV irradiation at 254 nm in a UV cross-linker (Hoeffer) for 90 s. RNA transcripts were then digested with 20 μg of RNase A (Sigma) for 40 min at 37 °C. The samples were separated over 15% SDS-PAGE. Gels were stained with Coomassie Blue, dried, and subjected to autoradiography or analyzed by phosphorimaging. In competition experiments unlabeled RNA transcripts were added in varying mass excess and preincubated with the protein samples before the addition of the radiolabeled probe. To examine how the redox state affected the binding proteins, GSSG or GSH was added to the protein extracts or to the purified Rubisco (25 ng) 10 min before the addition of the labeled 5′-UTR.

**Two-dimensional Gel Electrophoresis**—Protein separation on two-dimensional gel electrophoresis was performed as previously described (22). Separation on the first dimension was performed using ampholytes (Bio-Rad) that ranged between pH 3 and 9. Separation on the second dimension was preformed by SDS-PAGE over 12% polyacrylamide gels.

**Antiserum**—Polyclonal rabbit antisera raised against Rubisco holoenzyme from tobacco were a generous gift of T. J. Andrews from the Australian National University, Canberra, Australia.

**Western Blot Analysis**—Proteins were separated over one- and two-dimensional SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell). Western blot analysis was performed using anti-Rubisco antibodies (1:4000) and a conjugate of protein A with alkaline phosphatase (1:2000). Antibody binding was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) dissolved in a buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂.

**Rubisco Purification**—Wild type *C. reinhardtii* cells (CC-125) were grown in Tris acetate phosphate medium (3 liters), harvested to yield
Rubisco LSU (aa 1-150) was amplified by PCR using the LSU-fwd-(1) primer was 5′-TTCCACAAACTAATGTTTTAACG-3′. The DNA region that encodes the amino terminus of the rbcL gene, the LSU-fwd-(1-21) primer was 5′-ATCCCATGGAACCTCCA-CGGTATTC-3′, and the LSU-rev-(1506-1482) primer was 5′-TTAGGAATTCGTCGAAACT-3′. The DNA region that encodes for Rubisco LSU was amplified using primers derived from both ends of the large subunit of Rubisco were identified based on the SCOP data base that provides a structural classification of proteins (25).

Expression of Recombinant LSU and Its Subdomains in Bacteria—The DNA region that encodes for Rubisco LSU was amplified using primers derived from both ends of the rbcL gene; the LSU-fwd-(1-21) primer was 5′-ATCCCATGGAACCTCCA-CGGTATTC-3′, and the LSU-rev-(1506-1482) primer was 5′-TTAGGAATTCGTCGAAACT-3′. The DNA region which encodes the amino terminus of Rubisco LSU (aa 1-150) was amplified by PCR using the LSU-fwd-(1-21) primer; and the reverse primer, LSU-rev-(450-432), 5′-GGAAATTTCAACGTACAGTGTTTTAAG-3′. The DNA region that encodes for Rubisco LSU was amplified using the primer LSU-fwd-(451-466), 5′-ATCCCATGGAACCTCA-CTTAAACATCGATGTTTTAAG-3′. The DNA region that encodes for Rubisco LSU was amplified using primers derived from both ends of the gene was synthesized in vitro and incubated with the protein fractions. Binding of proteins to the radiolabeled RNA was monitored by their inhibitory effect on migration of the RNA in native polyacrylamide gels as compared with control unbound RNA. RNA binding activity was observed with the radiolabeled 5′-UTR of rbcL (Fig. 1A). Migration of the RNA-protein complexes resulted in multiple bands, suggesting that the binding involved more than a single protein. Binding to the labeled 5′-UTR of rbcL was interrupted in a dose-response manner by adding increasing amounts of the corresponding unlabeled rbcL 5′-UTR (Fig. 1A, lanes b-f). Binding was not affected by the addition of increasing amounts of SK RNA (lanes h-i) or by a large excess of E. coli tRNA (lanes m and n). Thus, only the homologous non-radioactive RNA fragment could efficiently compete out the RNA-protein interaction. In addition, no binding was observed with a nonspecific labeled RNA fragment of comparable size, derived from pBluescript (SK RNA, data not shown).

To strengthen the association between the shift of glutathione to its oxidized form during light stress and the translational arrest of Rubisco LSU (9), binding assays were carried out under oxidizing (GSSG) and reducing (GSH, DTT) conditions. Fractionated proteins capable of binding to the 5′-UTR of rbcL in EMSA were preincubated with increasing concentrations of GSSG, GSH, and DTT (5, 10, and 25 mM, respectively). The competition assay was performed with increasing concentrations of unlabeled rbcL 5′-UTR. Band shifting of the RNA was inhibited already in the presence of 5 mM GSSG and was completely abolished by 10 and 25 mM GSSG, whereas GSH and DTT had no effect on the binding activity (Fig. 1B). These results indicate that mediating the redox of thiol groups affects their ability to interact with the 5′-UTR of the rbcL transcript.
the binding was specific to the 5′-UTR of rbcL, UV cross-linking was performed in the presence of non-radioactive RNA competitors. Binding of the 81-, 62-, 51-, and 47-kDa proteins was inhibited by the homologous rbcL–93/+24 fragment (Fig. 2A, lanes a–g) and was hardly affected by the SK RNA or tRNA controls (Fig. 2A, lanes h–q), indicating a sequence specificity for the RNA-protein interactions. Because binding of the 38-, 34-, and 32-kDa proteins was not competed out by the cold rbcL RNA, their interaction with the RNA was most likely nonspecific.

**UV Cross-linking of Proteins to the 5′-UTR of rbcL Is Sensitive to the Redox State of Glutathione**—To test how the redox state of thiol groups modulated binding of proteins to the 5′-UTR of rbcL, UV-cross-linking assays were performed in the presence of reduced and oxidized glutathione. Protein extracts eluted from the heparin-Actigel column were preincubated with increasing amounts of GSSG before the addition of the labeled rbcL 5′-UTR. Under oxidizing conditions binding of the 81-, 62-, 51-, and 47-kDa proteins decreased (Fig. 2B); however, cross-linking of a new 55-kDa protein was observed. In view of the similar molecular weights of this newly UV-cross-linked protein and the LSU of Rubisco, the possibility that an autoregulatory pathway exists was considered.

**Rubisco LSU Is an RNA-binding Protein**—The 55-kDa protein characterized on one- and two-dimensional polyacrylamide gels combined with Western blot analysis. Proteins were preincubated with GSSG (7.5 mM) before their UV cross-linking with the radiolabeled 5′-UTR of rbcL. The cross-linked proteins were separated by two-dimensional SDS-PAGE and blotted onto nitrocellulose membranes. The blots were exposed to a film (Fig. 3, A and B) and then reacted with an antibody raised against Rubisco LSU (Fig. 3, A and B). Alignment of the autoradiograms and films indicated that the 55-kDa protein that cross-linked to the 5′-UTR of rbcL under oxidizing conditions co-migrated with Rubisco LSU on two-dimensional gels.

Direct evidence for binding of Rubisco LSU to the rbcL leader under oxidizing conditions was obtained by using the purified enzyme in UV-cross-linking experiments. As shown in Fig. 4, A and B, the purified protein cross-linked with the rbcL leader in direct correlation with the GSSG concentration, whereas no binding was observed in the absence of GSSG. In addition, the 55-kDa protein was absent in UV-cross-linking experiments performed under oxidizing conditions using CC 2653 (20), a mutant that fails to express Rubisco LSU due to a point mutation that pre-terminates translation (data not shown).

To examine the binding specificity between Rubisco LSU and its corresponding rbcL leader, competition assays were performed with unlabeled RNAs that corresponded to the homologous rbcL leader in its sense and antisense orientations. Equal inhibition of binding to the labeled rbcL leader was observed with similar amounts of either fragment, suggesting that in vitro the binding of Rubisco LSU to its leader was sequence-independent (Fig. 5A). A similar conclusion was drawn from experiments that compared the competition between non-labeled SK RNA and the sense rbcL fragment. Both of these RNA fragments competed out the binding between Rubisco LSU and its RNA leader with comparable efficiencies (Fig. 5B). It, therefore, appears that under oxidizing conditions Rubisco LSU binds nonspecifically to RNA. In the presence of a large excess of cold competitor RNA, a higher band was observed. Its appearance could result from incomplete RNase digestion that resulted in protein dimers held together by partially digested RNA fragments.

To further elaborate on the RNA binding features of Rubisco LSU in the presence of GSSG, UV-cross-linking experiments were performed with protein extracts eluted from the heparin-Actigel column and with purified Rubisco using various RNA templates. A similar binding pattern was obtained for three different chloroplast RNA leaders, rbcL, psbA, and atpB, under reducing (DTT) conditions. Apparently, under oxidizing conditions, the 55-kDa Rubisco LSU also bound to the three leaders (Fig. 6A). Similarly, purified Rubisco LSU cross-linked with all the RNA templates tested, including the leaders of rbcL in the sense and antisense orientation, the leaders of psbA and atpB (chloroplast-encoded genes), α-tubulin (a nuclear gene), and the plasmid-derived SK RNA (Fig. 6B). These findings indicate that oxidized Rubisco LSU could interact with any RNA fragment in a sequence-independent manner.

To examine whether the interaction of oxidized LSU with RNA was conformation-dependent, UV cross-linking was performed with heat-treated Rubisco. The enzyme was incubated in gradually increasing temperatures (25–100 °C) for 10 min before UV cross-linking with the rbcL leader. When Rubisco was first oxidized by the addition of GSSG and then heated, binding to RNA was hardly affected up to 80 °C. However, if Rubisco was first heated and then oxidized by GSSG, temperatures as low as 37 °C affected its ability to bind RNA. Moreover, when the protein was first heated and then oxidized, UV cross-linking revealed a larger molecular species that could have evolved from cross-linking between different denatured...
subunits (Fig. 7). The larger labeled proteins were not observed if Rubisco was first oxidized and then heated. A possible explanation of this observation is that upon oxidation, vicinal thiol groups interact to form S-S bridges that stabilize the domain that binds RNA, even after exposure to elevated temperatures.

The Putative RNA Binding Domain of Rubisco LSU.—To understand the structural basis for the RNA binding activity observed in Chlamydomonas Rubisco LSU, we tried to gain some insight from its crystal structure (26, 27). The crystal structure of Rubisco consists of two domains, a TIM barrel carboxyl-terminal domain (aa 151–475) and a smaller amino terminus domain (aa 1–150). The working hypothesis was that the RNA binding site of Rubisco is likely to share some structural similarity with known RNA binding proteins. Using SCOP, a data base that provides a structural classification of proteins (25), we were able to identify considerable structural similarity between the amino terminus (residues 1–150) of Rubisco and a number of RNA-binding proteins (28). Both the LSU amino terminus and these RNA-binding proteins have the βαβαβαβ structure known as “ferredoxin-like” domain. Fig. 8A shows the structural similarity between the RNA binding domain of the human splicing factor U1A (Protein Data Bank code 1drz, residues 10–87) in complex with the hepatitis δ virus ribozyme (26) and the amino terminus of Rubisco LSU (Protein Data Bank code 1gk8, residues 38–139) (29). The root mean square deviation of 39 Cα atoms (colored) comprising five of the six secondary structure segments is 2.6 Å. Unlike the amino terminus, no such similarity was found between the carboxy terminal domain of LSU and any known structure of an RNA-binding protein. Thus, we hypothesized that the RNA binding site of the LSU is located within its amino-terminal domain.

To further examine whether the amino terminus of Rubisco LSU (aa 1–150) can bind RNA, the gene encoding this protein domain was cloned and expressed in bacteria. The binding affinity of the Rubisco holoenzyme and the truncated LSU (aa 1–150) to RNA was determined by UV cross-linking performed with increasing protein concentrations in the presence of excess radiolabeled target RNA (Fig. 9A). The dissociation constant of Rubisco holoenzyme was 9.9 nM for the 5′-UTR of rbcL and 15.9 nM for SK RNA. The dissociation constant of the recombinant 1–150 polypeptide for SK RNA was 7.9 nM. The measured 

Fig. 3. The 55-kDa protein that UV cross-links with the rbcL leader comigrates with Rubisco LSU on two-dimensional gels. The [α-32P]UTP-labeled 5′-UTR of rbcL was incubated with protein extracts under normal and oxidizing conditions (0 and 7.5 mM GSSG, respectively). The samples were UV-cross-linked, and the mixtures were digested with RNase A. The proteins were then separated on two-dimensional gels and blotted onto nylon membranes. The blots were reacted with antibodies against Rubisco (A and B) and autoradiographed (C and D). The bars and autoradiograms were aligned to determine the position of Rubisco LSU.

Fig. 4. Binding of purified Rubisco LSU to the rbcL 5′-UTR is sensitive to GSSG concentrations. A, purified Rubisco was preincubated with increasing concentrations of GSSG before the addition of the [α-32P]UTP-labeled rbcL 5′-UTR. The samples were UV-cross-linked, treated with RNase A, and resolved on 15% SDS-PAGE. B, Coomassie Blue staining of the cross-linked proteins showing equal protein content in the different lanes.

Fig. 5. Rubisco LSU binds RNA in a sequence-independent manner. A, competition with unlabeled sense and antisense rbcL RNAs. Purified Rubisco (25 ng) was pretreated with 2 mM GSSG and incubated with increasing amounts of competing non-labeled RNA fragments comprising sense (rbcL) or antisense (as-rbcL) RNAs before the addition of the radiolabeled rbcL 5′-UTR. The samples were UV-cross-linked, treated with RNase A, and resolved on 15% SDS-PAGE. B, competition with SK RNA and with the sense rbcL RNA. Purified Rubisco (25 ng) was pretreated with 2 mM GSSG and incubated with increasing amounts of competing non-labeled RNA fragments comprising the sense (rbcL) or SK RNA before the addition of radiolabeled rbcL 5′-UTR. The samples were treated as described in A.
The effect of GSSG on binding to RNA was examined (Fig. 9B) for the recombinant LSU (aa 1–475) and the truncated protein fragments corresponding to amino acids 1–150 (amino terminus) and 151–475 (TIM barrel structure). The purified recombinant polypeptides reacted with antibodies raised against Rubisco. The amino terminus (aa 1–150) gave two bands in Western blot analysis, possibly due to proteolytic cleavage that occurred within the bacteria. The recombinant LSU (aa 1–475) cross-linked to RNA mainly in the presence of GSSG, as also observed with the holoenzyme (Fig. 4). However, binding of the amino-terminal domain (aa 1–150) occurred with and without GSSG. When this protein fragment was independently expressed in bacteria it was exposed, allowing RNA binding. The addition of GSSG even impeded this activity. The TIM barrel domain showed no binding of RNA in the presence or absence of GSSG.

Examination of the crystal structure of Rubisco (26, 27) indicates that the amino terminus is not exposed to the surface of the protein and is covered by the TIM barrel structure (Fig. 8, B and C). However, if the amino-terminal domain is responsible for the RNA binding activity, it should be exposed to the surface. Because oxidative stress leads to structural changes in Rubisco (31), these could result in exposure of the amino terminus domain, making it accessible to RNA.

**DISCUSSION**

Assembly of protein complexes on the 5′-UTRs of chloroplast genes is a key process in translational regulation (32–34). Previous studies indicate that the interaction between RNA-binding proteins and the 5′-UTR of psbA and atpB are subject to regulation by the redox state of the electron carriers, mainly thioredoxin (16, 32). Our studies on rbcL revealed a novel regulatory pathway that leads to translational arrest of the large subunit of Rubisco during light-induced oxidative stress. Rather than sensing the redox state of the electron carriers in the photosynthetic chain, translation of Rubisco LSU responds to accumulation of ROS in the chloroplast (9). Light-induced oxidative stress caused a shift in the glutathione pool toward its oxidized form, and during that period translation of Rubisco LSU was arrested. Once the cells reduced their chlorophyll content and recovered from their photooinhibitory state, the level of ROS decreased, and the original GSH/GSSG ratio was regained. In an attempt to understand this unique pattern of regulation, we investigated potential regulatory routes based on RNA-protein interactions that were affected by the redox state of glutathione.

Using EMSA, we show that the RNA-binding proteins assemble on the 5′-UTR of the rbcL transcript. The specificity of this binding was established by competition assays using the homologous rbcL leader and non-related RNA competitors. UV-cross-linking experiments with the radiolabeled 5′-UTR of rbcL revealed a group of proteins that bound under reducing conditions, with molecular masses of 81, 62, 51, 47, 38, 34, and 32 kDa. However, only binding of the 81-, 62-, 51-, and 47-kDa proteins was competed out by the homologous cold RNA and not by nonspecific competitors such as SK RNA or tRNA, indicating their binding specificity. A similar pattern of binding was obtained for the 5′-UTRs of rbcL, psbA, and atpB, suggesting that these proteins bound universally to chloroplast leaders. These results are in line with previous reports describing proteins sized 81, 62, 51, 47, and 15 kDa that interact with the rbcL leader as well as with other chloroplast leaders, including psbA, atpB, rps7, and rps12 (17). In a more recent study Zerges et al. (19) show, using competition experiments, that several membrane proteins of similar sizes (80, 60, 46, 47, and 30 kDa) have affinity to AU-rich RNAs, and their binding increases by light and by modulation of the ADP pool.

The potential role of changes in the redox state of sulfhydryl groups in modulating binding of proteins to the rbcL 5′-UTR was obtained both by EMSA and UV-cross-linking experiments carried out under oxidizing (GSSG) and reducing (GSH) conditions. Increasing concentrations of GSSG decreased the binding activity of fractionated proteins to the rbcL 5′-UTR as compared with binding under reducing conditions. These results corroborate our hypothesis that modulation of the intrachloroplastic redox potential regulates translation of Rubisco LSU by modulating the redox state of thiol groups on RNA-binding proteins.

UV-cross-linking experiments showed that binding of the 82-, 62-, 51-, and 47-kDa proteins was interrupted under oxidizing conditions, and a new protein of 55 kDa that co-migrated with Rubisco LSU was cross-linked to the RNA, as revealed in one- and two-dimensional gels (Fig. 3). Labeling of the 55-kDa protein increased in correlation with GSSG concentrations and reached saturation within the physiological range (5–10 mM (5)). To further verify the identity of the 55-kDa protein, UV-cross-linking experiments were performed with purified Rubisco under normal and oxidizing conditions. LSU from purified Rubisco bound to its corresponding leader under oxidizing conditions (GSSG). In addition, the protein was absent in extracts prepared from a mutant that fails to express Rubisco LSU. However, competition experiments indicated that the binding was not sequence-specific, since both sense and antisense RNAs derived from the 5′-UTR of rbcL competed-out the binding of purified Rubisco LSU to its leader (Fig. 5), and a similar effect was observed with SK RNA. Furthermore, we found that under oxidizing conditions, purified Rubisco LSU bound to a variety of RNA leaders derived from both chloroplast and nuclear genes as well as to a random RNA fragment (pSK).

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Synthesis of protein subunits that assemble into larger complexes in the chloroplast is subject to autoregulation processes that coordinate the level of the subunits. The carboxyl terminus of the unassembled cytochrome \( f \) subunit interacts with the 5′-UTR of \( petA \) gene and inhibits its translation (35). Feedback repression was also reported for Rubisco biogenesis. When expression of the small subunit of Rubisco was eliminated by deletion mutagenesis in \( C. reinhardtii \) (36) or by transformation of tobacco plants with antisense \( rbcS \) RNA, synthesis of the corresponding LSU was inhibited (37). The mechanism that controls expression of Rubisco LSU in the absence of the eight small subunits has not yet been clearly determined. However, an autoregulatory pathway involving unassembled LSU could inhibit its own synthesis. Alternatively, the presence of unassembled eight small subunits could be required for inducing translation of LSU (36). In this study we show that Rubisco LSU has the structural characteristics of an RNA-binding protein; however, an autoregulatory pathway could not be established, since binding of RNA occurs \textit{in vitro} in a sequence-
independent manner. At this stage we cannot rule out the other protein yet unidentified, directing a sequence-specific binding that causes the translational arrest. Such aided binding was reported for CYT-18, the mitochondrial tyrosyl-tRNA synthetase in *Neurospora crassa*. CYT-18 functions in the splicing of group I introns by inducing a catalytically active RNA structure. Its interaction with RNA is mediated by CYT-19, an ATP-dependent RNA chaperone which destabilizes non-native RNA structures that constitute kinetic traps in the CYT-18-assisted RNA folding pathway (38). Analysis of the binding pattern of proteins to different chloroplast 5′-UTRs gave the same pattern under reduced and oxidizing conditions. However, this does not rule out the involvement of a gene-specific regulatory protein, in line with recent reports on the role of specific regulators that control expression of several chloroplast genes. For example, Nac2 is required for synthesis of psbD in *C. reinhardtii*. Although it comigrates with high molecular weight non-polysomal complexes containing RNA, there is no evidence for a direct interaction between Nac2 and its own RNA (39). Other examples are the Tbc proteins 1–3, which control expression of psbC (40, 41), and Mbh1, which is required for expression of the chloroplast psbB/psbP/psbH gene cluster (42).

The crystal structure of Rubisco has been previously solved (26, 27), yet no RNA binding motifs were identified. The large subunit of the protein consists of two domains, a TIM barrel carboxyl-terminal domain (aa 151–475) and a smaller amino terminus domain (aa 1–150) that is classified as a ferredoxin-like domain by SCOP (25). The amino terminus is remarkably similar to a consensus structure shared by a diverse set of RNA-binding proteins containing the so-called RNA binding domain (RBD) (Fig 8A). The RBD is composed of a 150 aa–βββ secondary structure pattern that forms a four-stranded anti-parallel β sheet packed against the two perpendicularly oriented α helices (28, 43, 44). The RNA binds to the solvent-exposed face of the β sheet. The analogy to the RBD-containing proteins suggests that the binding site is the interface between the amino terminus β-sheet and the 354–364 loop of the carboxyl terminus domain (Fig. 8B). If the binding site indeed occurs at the interdomain interface, the formation of disulfide bonds during oxidation stress may expose this region and make it accessible to RNA. A possible candidate for this role is the 172–192 pair of adjacent, but non-bonded cysteine residues, buried within the holoenzyme. These have already been reported to mediate redox regulation of other Rubisco activities (31, 45). Involvement of other cysteine residues cannot be ruled out at this stage. A recombinant polypeptide that encompasses the predicted RBD was capable of binding RNA in *vitro*, with a binding constant that was similar to that measured with purified Rubisco and in accordance with $K_V$ values reported for other RNA binding proteins (30, 46). Furthermore, binding of the recombinant amino-terminal domain did not depend on the redox state in the binding reaction, in agreement with our suggestion that under reducing conditions interaction with the RNA is hindered by steric interference of the carboxyl-terminal domain.

The physiological role for the RNA binding activity of Rubisco is intriguing. As indicated, it could have a specific function in an autoregulatory loop, possibly aided by additional protein factors. Alternatively, its affinity for RNA could serve a broader function. Various proteins, such as viral polypeptides (47) or the cold stress chaperone CspA (48), can bind RNA in a sequence-independent manner. It was suggested that CspA functions as an RNA chaperone that binds to RNA during cold stress. This binding may prevent the formation of secondary structures that make the RNA susceptible to nucleolytic cleavage. Thus, it was suggested that CspA may protect processes that involve RNA, including transcription and translation (49). We speculate that Rubisco LSU could play a similar protective role. It was reported that during oxidative stress, Rubisco translocates itself from the stroma into the chloroplast membrane, where translation of chloroplast proteins takes place (18). Thus, it is possible that oxidized Rubisco LSU could serve as an RNA chaperone (50) that protects RNA from damages that occur during oxidative stress.

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42. Vaistij, F. E., Boudreau, E., Lemaire, S. D., Goldschmidt-Clermont, M., and Rochaix, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 14813–14818
43. Burd, C. G., and Dreyfuss, G. (1994) *Science* **265**, 615–621
44. Siomi, H., and Dreyfuss, G. (1997) *Curr. Opin. Genet. Dev.* **7**, 345–353
45. Moreno, J., and Spreitzer, R. J. (1999) *J. Biol. Chem.* **274**, 26789–26793
46. Severson, W., Partin, L., Schmaljohn, C. S., and Jonsson, C. B. (1999) *J. Biol. Chem.* **274**, 33732–33736
47. Lopez, C., Navas-Castillo, J., Gowda, S., Moreno, P., and Flores, R. (2000) *Virology* **269**, 462–470
48. Karlson, D., Nakaminami, K., Toyomasu, T., and Imai, R. (2002) *J. Biol. Chem.* **277**, 35248–35256
49. Bae, W., Xia, B., Nourse, M., and Severinov, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7784–7789
50. Cristofari, G., and Darlix, J. L. (2002) *Prog. Nucleic Acids Res. Mol. Biol.* **72**, 223–268
RNA Binding Activity of the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Large Subunit from *Chlamydomonas reinhardtii*

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